THE ROLE OF ERα IN THE UTERUS DURING EMBRYO IMPLANTATION AND IN THE OVARY DURING OVARIAN TUMORIGENESIS

BY

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DISSERTATION

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ABSTRACT

The molecular actions initiated by the steroid hormone estrogen elicit a multitude of biological effects in physiological and pathophysiological processes. Estrogen acts via its cognate steroid hormone receptor, estrogen receptor alpha (ERα) or beta (ERβ), to fulfill these biological processes. The studies reported here investigate estrogen's action via ERα in uterine physiology and ovarian pathophysiology and is written in two parts. The first part is an investigation of ERα and its downstream target Hif2α in the uterus during embryo implantation. The later part of the dissertation discusses the role that ERα plays in ovarian tumorigenesis.

Both humans and rodents exhibit hemochorial placentation, an invasive form of embryo implantation which is characterized by intimate contact of the implanted embryo with maternal blood. Implantation is initiated when the embryo first attaches to the uterine epithelial cells and invades through these cells to the underlying stroma. This process triggers the uterine stromal cells to undergo proliferation and differentiation to form the decidua, a secretary maternal tissue that provides the necessary nutrients to support embryonic life prior to placentation. Through the development of knockout mouse models, it is known that ERα, the predominant estrogen receptor in the uterus, is essential for the embryo to attach to these cells prior to invasion. However, we have uncovered for the first time the essential role of ERα in uterine stromal cell differentiation during embryo implantation. We have developed a conditional knockout mouse model, termed $ER\alpha^{d/d}$, which has ERα deleted in all cell types which express progesterone receptor (PR). By employing artificially induced decidualization we know that ERα in the uterine stromal cells is essential for proliferation and decidualization.

To date very few molecules have been shown to be regulated by estrogen in the uterine stromal cells that are essential for decidualization. Our laboratory has shown that it is local estrogen production acting via ERα within the uterine stromal cells that drives and sustains the decidualization response. Furthermore this local estrogen production regulates endothelial PAS domain protein 1 (EPAS1), also called hypoxia-inducible factor 2 alpha (Hif2α). Hif2α is a transcription factor that has been shown to be regulated by hypoxic conditions and regulates molecules essential for an angiogenesis response. In order to study the functional role of Hif2α, we have created a conditional knockout of the Hif2α gene which has Hif2α knocked out

in all cells expressing PR. This conditional knockout mouse, termed Hif2α$^{d/d}$, is infertile. During pregnancy embryos travel to the uterine lumen and attach to uterine epithelial cells. This attachment elicits an initial decidual response. This is the first *in vivo* model to show that embryo attachment and not embryo invasion through the epithelial cells provokes a stromal cell decidual response. In absence of Hif2α, stromal cells begin the process of decidualization and display alkaline phosphatase activity, a marker of differentiation. However, prolactin related protein (PRP), a decidual marker induced later in the decidualization process, is absent in the Hif2α$^{d/d}$ uterus indicating that the terminal differentiation of stromal cells is compromised in absence of Hif2α. Furthermore, without Hif2α in the stromal cells, angiogenesis at the early stages of pregnancy is impaired as marked by a downregulation of an endothelial cell marker, PECAM. Understanding downstream molecules of Hif2α will undoubtedly give insight into the molecular pathways essential for implantation. The role that uterine ERα and its downstream target, Hif2α, plays in the early phases of pregnancy will be the topic of chapter 2.

The second part of this dissertation focuses on the role that ERα plays in the pathophysiology associated with ovarian tumorigenesis. We have generated an animal model which forms epithelial ovarian tumors with 100% penetrance. This animal model is a conditional knockout for the ERα, termed ERα$^{d/d}$. This animal shows a lack of ERα expression in the pituitary which leads to a lack of negative estrogen feedback on the hypothalamus-pituitary-ovarian axis resulting in high serum levels of luteinizing hormone, estradiol, progesterone and testosterone. Interestingly, ovarian expression of ERα in this animal model remains intact. We have shown that by treating animals with letrozole, an aromatase inhibitor, that ovarian tumor volume is decreased compared to untreated animals, indicating a novel role of ERα in ovarian tumorigenesis. This animal model mimics the genetic profile of human serous epithelial ovarian carcinoma and provides a novel tool to study epithelial ovarian tumorigenesis for two reasons: 1) tumors form slowly over the course of the animal's life mimicking human epithelial ovarian tumors and 2) ovarian tumors form due to a physiological perturbation and not a mechanical perturbation, such as intra-bursal injection to induce oncogene expression, further mimicking the human ovarian tumor formation. This animal model will aid in our understanding of how epithelial ovarian tumors form and will likely lead to the discovery of treatments and/or diagnostic tools for this disease.

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Part I: The Role of ERα and Its Downstream Target, Hif2α, in the Uterus During Embryo Implantation

CHAPTER 1:Literature Review - Part I

The American College of Obstetricians and Gynecologists (ACOG) estimates that 15% of all known human pregnancies will end in miscarriage. The actual number of failed pregnancies is probably significantly higher since many miscarriages or early pregnancy loss is not detected by the mother. Due to extensive research in the area of pregnancy, the human population is now aware of factors which increase the risks of pregnancy loss such as alcohol consumption or cigarette smoking by the mother. However, molecular players associated with pregnancy loss remain unclear.

To better understand why miscarriages occur, the process of implantation and stages leading to the establishment of pregnancy need to be further investigated. Assisted reproductive technologies (ART) have become the method of choice for those who have recurring miscarriages. Undoubtedly with a better understanding of the implantation process, the success of ART could be enhanced significantly.

Ethically, the scientific analysis of human pregnancy remains a difficult subject of research. We therefore, rely on animal models that mimic the pregnancy process of the human to better understand the complexity of this phenomenon.

Although the process of implantation and placentation varies greatly among mammalian species, the human and rodent exhibit hemochorial placentation. In these species, implantation is invasive in nature allowing for the maternal and fetal tissues to come into intimate contact with each other (1-2). We therefore use the mouse as an animal model to study the processes of implantation and pregnancy. Furthermore, due to the power of genetics, this animal model also allows us to explore the function of target molecules *in vivo* by using knockout strategies. Part I of this dissertation discusses molecular pathways underlying the physiology of pregnancy in the mouse.

1.1 Implantation

Implantation of the embryo into the uterine wall occurs in three distinct steps: apposition, adhesion, and invasion. In the mouse after the oocyte is released from the ovary, fertilization begins in the oviduct when the sperm meets the egg. The fertilized egg will then take 4 days for the journey through the oviduct into the uterus where the implantation process will begin.

The embryo will first become closely apposed to the luminal epithelium of the uterus beginning on day 4 of pregnancy. We denote day 1 of pregnancy as the day in which we see the copulatory plug. The next phase is termed adhesion. At this point, the trophectoderm becomes tightly adhered to the luminal epithelium. The invasive phase of implantation occurs in the third stage of the process. At this time, the trophectoderm will penetrate the luminal epithilium and progress into the uterine stromal tissue (3-5). Subsequently, the implanting embryo will initiate stromal cell proliferation and differentiation into secretory decidual cells, a process termed as decidualization.

It is known that implantation and decidualization are under direct control of steroid hormones estrogen (E) and progesterone (P). In the mouse, there is a "nidatory surge" of ovarian E on day 4 of pregnancy (6-7). This surge of E has been shown to be critical for the initiation of implantation. Experimentally, this phenomenon has been clearly established in the delayed implantation mouse model. In this model, if pregnant animals are ovariectomized on day 4 morning, thereby removing the circulating steroid hormones, the embryo will not implant. Progesterone is essential to prepare the uterus to receive the implanting embryo. If P is given the embryo will be maintained in the free-floating state within the uterine lumen. However, if several days of P administration are followed by only a single dose of E, the embryo will immediately begin the implantation process (8-9). This experimental model is a convenient system to study the downstream targets of E action in the early stages of implantation.

Implantation occurs in the presence of the correct hormonal milleu, termed as the "window of implantation". Under the influence of E and P, the uterus will be in one of three phases of receptivity: neutral, receptive, and refractory. During delayed implantation, administration of P will leave the uterus in a neutral state. In the presence of E, the uterus will

become receptive and the implantation process will begin. However 24-36 hours after the administration of E, the uterus will become refractory and implantation will no longer occur (10-11).

1.2 Decidualization

Following embryo apposition, adhesion, and invasion through the luminal epithilium, the process of decidualization is initiated. The invasion of the embryo into the uterus leads to several rounds of stromal cell proliferation followed by differentiation (12). The resulting tissue, termed the decidua, exhibits morphologically and biochemically different traits than the originating stromal cell. The decidua is thought to secrete hormones and cytokines necessary for implantation as well as provide an immune barrier to the mother from the allogenic embryo (13-15).

The embryo implants into the anti-mesometrial area of the uterus and invades toward the mesometrial area. During implantation, the anti-mesometrial area will form a primary decidual zone surrounding the embryo on day 5 of pregnancy and is initially devoid of blood vessels. The decidualization will span into a secondary decidualization zone beginning on day 6 of pregnancy forming the decidua capsularis (7). In the mesometrial region, the differentiated stroma will become the decidua basalis. The decidua eventually will be replaced by the placenta.

The proliferation and differentiation of stromal cells during decidualization is also regulated by steroid hormones E and P (16). We can analyze the decidualization process *in vivo* in absence of the embryo using the artificial decidualization model system (ADR). In this experimental model, non-pregnant animals are ovariectomized followed by a priming of E. Estrogen levels are critical to prepare the uterus for decidualization (17). Animals will then be administered either P or E plus P followed by a stimulus, such as oil injection into the uterine lumen, to induce decidualization (18). In this experimental model blockage of progesterone receptor (PR) and estrogen receptor (ER) activities by anti-progestins or anti-estrogens will

impair decidualization indicating crucial roles of PR and ER in the regulation of this process (19).

1.3 Angiogenesis of Decidua

Development of the mammalian embryo depends on the successful initiation of implantation, formation of decidua followed by placentation. The formation of the hemochorial placenta, common to both the rodent and human, consists of three layers. The outer layer is of maternal origin and is composed of decidual cells of the uterus. This layer contains maternal vasculature that brings blood to/from the implantation site. The middle region is termed the "junctional" area. This area is where the fetal placenta attaches to the uterus. The inner layer is composed of highly branched villi that are designed for efficient nutrient exchange. This area is also known as the labyrinth layer and is derived from a trophoblast lineage (2). The villi will be bathed in maternal blood in order to facilitate transfer of nutrients.

The formation of a mature placenta is not evident until E12.5. The first step in labyrinth formation is the chorioallantoic attachment and this occurs at E8.5 (2). At this point fetal blood vessels will begin to grow. Prior to the chorioallantoic attachment, the maternal decidua must be fully vascularized in order to support the growing embryo.

In the mouse, a conclusive sign of implantation initiation is an increased uterine vascular permeability at the sites of blastocyst apposition. This heightened localized vascular permeability can be visualized by discrete blue bands using an intravenously injected blue dye at the time of embryo attachment and is considered one of the earliest prerequisite events for the process of implantation. Vascular endothelial growth factor (VEGF), a potent endothelial cell mitogen that mediates rapid endothelial proliferation, is expressed in the decidua on day 5 morning during the initiation of vascular permeability (26). It is widely believed that VEGF plays a key role in angiogenesis within the uterine decidua during pregnancy (27-29).

During decidualization, endothelial cells are recruited to the deciduas, initiating angiogenesis (20). The establishment of a vascular network in the decidua is essential for embryonic growth. It is known that steroid hormones E and P play critical roles in the process of angiogenesis; however, their specific roles in this process remain controversial. Reports have shown that E increases vascular permeability, but does not increase endothelial cell proliferation (21). In contrast, others observed a rapid induction of endometrial endothelial cell proliferation within 24 h of E treatment in ovariectomized mice, suggesting a rapid angiogenic response (22). Contradicting this observation, Ma et al. concluded that E promotes uterine vascular permeability but profoundly inhibits angiogenesis, whereas P stimulates angiogenesis with little effect on vascular permeability (23). The difference in experimental models used in these studies may be a cause of inconsistency concerning the issue of steroid hormone regulation of angiogenesis prior to placentation.

We are interested in understanding the role that E plays in the regulation of angiogenesis prior to placentation. Using an *in vivo* model of artificially delayed implantation, we have clearly established that local production of E in the uterine stromal cells promotes decidualization and angiogenesis prior to placentation (24). By treating mice with aromatase inhibitor, Das et al. show that inhibiting local production of E reduces angiogenesis in the decidua and impairs expression of angiopoietins. Furthermore we have shown that Connexin 43, a gap junction protein, is regulated by estrogen during implantation and is critical for angiogenesis in the decidua (25). These studies have clearly established a role of E in angiogenesis of the decidua prior to placentation.

1.4 Estrogen Receptor Alpha (ERα) Function in the Uterus during Early Pregnancy

The molecular actions of the steroid hormone E are mediated by the cognate steroid hormone receptors, estrogen receptors alpha and beta. Estrogen receptors act as transcription factors to regulate down-stream target genes. Global ERα knockout mice are infertile and have hypoplastic uteri (30). Exposure to estrogen fails to induce uterine hypertrophy in ERα knockout

mice. Furthermore embryo transfer experiments indicate that uterine ERα is essential for embryo implantation (31). In contrast ERβ knockout mice are subfertile, but can carry pregnancies to term and their uteri respond to estrogen treatment (32). The cause of infertility in these mice is a result of ovarian dysfunction. Evidence from these knockout models clearly indicates that ERα is the predominant regulator of estrogen function in the uterus.

Prior publications have concluded that ERα is essential for embryo implantation, but is indispensible for uterine decidualization (31). These studies were carried out using an ERα global knockout mouse which forms alternative splice variants of the ERα gene (30, 33). Alternative splice variants of ERα in the knockout mouse could be functional. Therefore, the role of ERα in uterine stromal cell differentiation needs to be reexamined using alternative models.

The transcription factor ERα classically acts by binding to estrogen response elements (EREs) in the promoter region of target genes. Alternatively, ERα has the ability to bind to other transcription factors to regulate target genes, a molecular event termed the non-classical tethering mechanism of ERα. Generation of a non-classical receptor knock-in mouse allows an investigator to pinpoint the transcription mechanism of ERα signaling during physiological processes *in vivo* (34). This knock-in animal model lacks the ability of ERα to bind directly to EREs. In order to determine downstream targets of ERα in uterine function, it is imperative to determine by which of these genomic transcription mechanisms ERα acts.

1.5 Hypoxia-inducible Factor 2 alpha (Hif2α)

Hypoxia-inducible factor 2 alpha (Hif2α), also termed endothelial PAS domain protein 1 (EPAS1), is a transcription factor that binds to hypoxia response elements in genomic DNA and is known to regulate genes involved in erythropoiesis, angiogenesis, metastasis, and proliferation (35). Hif2α is thought to be constitutively expressed and regulated by post-transcriptional mechanisms involving hypoxia (36). Under normoxic conditions, Hif2α is targeted for

proteosomal degradation, but stabilized under hypoxic conditions. This stabilized alpha subunit then binds to its beta counterpart, ARNT, and becomes transcriptionally active.

Hif2α has been shown to be present in the mouse uterine stromal cells. Even though classical regulation of protein accumulation is thought to be mediated by oxygen levels, previous reports have shown that estrogen regulates mRNA levels in uterine stromal cells (37). Peak induction of Hif2α occurs 6 hours post estrogen treatment in animals that have been ovariectomized. Furthermore, our laboratory has shown that local estrogen production by uterine stromal cells induces Hif2α mRNA expression. These findings lead us to believe that Hif2α could provide a novel insight into estrogen's regulation of angiogenesis within the decidua prior to placentation.

1.6 Specific Aims

Aim 1. To investigate the role of ERα and its downstream targets in endometrial stromal cell decidualization and angiogenesis:

a. Elucidate the functional role of ERα in endometrial decidualization and angiogenesis during early pregnancy by generating a conditional knockout of ERα.

b. Analyze the reproductive phenotypes of the ERα knockout females.

Aim 2. Investigate the function of Hif2α in murine uterine function:

a. Elucidate the functional role of Hif2α, a target of E, in endometrial angiogenesis during early pregnancy by generating a conditional knockout of Hif2α.

b. Analyze the reproductive phenotypes of the Hif2α knockout females.

CHAPTER 2: The Role of Estrogen Receptor Alpha and its Down-stream Target Hypoxia-inducible Factor 2 Alpha in Uterine Function during Early Pregnancy

2.1- Abstract

During early pregnancy, the concerted actions of the steroid hormones estrogen (E) and progesterone (P) via their cognate receptors orchestrate the changes in the uterine epithelium that make it competent to attach to the blastocyst and initiate the process of implantation. It is followed by the proliferation and differentiation of the underlying stromal cells into morphologically and functionally distinct decidual cells, which control trophoblast invasion and embryonic growth. Concomitant with this cellular transformation, known as decidualization, an extensive vascular network develops in the stromal compartment that supports the development of the implanted embryo. Although P acting via the progesterone receptor (PR) is essential for decidualization, the role of E in this process is less clear. To address this issue, we used the Cre-Lox strategy to create conditional mutant mice in which estrogen receptor alpha (ERα) expression is abolished in the uterus. Strikingly, the uteri of these mutant mice failed to show any response to a decidual stimulus, revealing for the first time a critical role of ERα in stromal decidualization. Subsequent investigations indicate ERα in the stroma is critical for cell proliferation, differentiation and angiogenesis during decidualization. It is known that local estrogen produced by uterine stromal cells drives stromal cell differentiation and vascular network formation. Treating mice with aromatase inhibitor indicate that this local E production regulates the expression of hypoxia-inducible factor 2 alpha (Hif2α), a transcription factor known to promote angiogenesis in other tissues. Hif2α is widely expressed in uterine stromal cells during decidualization and is induced by E in the ovariectomized mouse model. To address the functional role of this protein, we used the Cre-Lox strategy to create conditional mutant mice in which Hif2α expression is abolished in the uterus. These transgenic mice maintain the ability to ovulate but are completely infertile revealing for the first time a critical role of Hif2α in the uterus in maintaining pregnancy. Further histological examination indicates that embryos attach to the uterine luminal epithelial cells of Hif2α conditional knockout mice but fail to penetrate through the epithelium on days 5 and 6 of pregnancy. Despite the lack of embryo invasion, uterine stromal cells in Hif2α null mice differentiate as marked by alkaline

phosphatase activity, an early marker of decidualization. This remarkable phenotype shows for the first time that embryo attachment, and not embryo invasion, initiates stromal cell decidualiation. Subsequent analysis shows that the decidua of Hif2α conditional knockout mice fail to express prolactin-related protein, a terminal marker of decidualization, indicating that stromal cells are not able to fully differentiate. Furthermore, the Hif2α conditional knockout uterus fails to form a vascular network within the decidua on day 7 of pregnancy. Collectively, these results indicate a novel role for uterine ERα and its downstream target Hif2α in the regulation of mouse embryo invasion into the stromal compartment, decidualization as well as angiogenesis within the decidua prior to placentation.

2.2- Introduction

Embryo implantation is regulated by the concerted actions of steroid hormones E and P. These hormones act via their individual cognate steroid hormone receptors. Estrogen receptor alpha (ERα) is the predominant estrogen receptor in the uterus. Studies involving ERα global knockout mice have shown that uterine ERα mediates embryo attachment to the uterine luminal epithelium. Subsequent studies using ERα global knockout mice have concluded that ERα is dispensable for uterine stromal cell decidualization following embryo implantation (31). Kos et al. have determined that the ERα global knockout mice used in these studies express alternative splice variants of the ERα gene raising the possibility that a functional form of ERα remains in these knockout mice (33). Therefore, the role of ERα in uterine stromal cell decidualization warrants further investigation.

Recently we reported that local estrogen production by uterine stromal cells is essential for stromal cell differentiation and angiogensis within the decidua prior to placentation (24). We hypothesize that the molecular actions of local estrogen production are mediated by uterine stromal cell ERα. To address the functional role of ERα, we generated a conditional knockout mouse. ERα mice with loxp sites flanking the exon 3 of the ERα gene, ERα floxed mice, were generated by Dupont et al. (39). These flox mice were crossed with mice expressing cre-

recombinase driven by the endogenous progesterone receptor (PR) (38). The conditional knockout mice generated are termed, ERα$^{d/d}$ mice. Using this recently developed mouse model we show that ERα plays an essential role in uterine stromal cell proliferation, decidualization as well as angiogenesis. Identifying down-stream targets of ERα in uterine stromal cells is essential to understand embryo implantation and uterine angiogenesis.

Previous reports have shown that E regulates the expression of hypoxia-inducible factor 2 alpha (Hif2α) in uterine stromal cells (24, 37). Hif2α is a transcription factor known to induce expression of genes essential for angiogenesis. Because Hif2α is regulated by estrogen during pregnancy this protein could be the molecular link pinpointing estrogen action in promoting uterine angiogenesis. Our initial immunohistochemical studies indicate that Hif2α is expressed in the nucleus of uterine decidual cells during pregnancy. To study the functional role of Hif2α, we created conditional knockout mice by crossing mice harboring loxp sites surrounding exon 2 of the Hif2α gene with mice expressing cre-recombinase knocked in under the endogenous PR. The Hif2α conditional knockout mouse is able to ovulate but is infertile. The studies described in this chapter identify Hif2α, a down-stream target of ERα, as an essential protein for embryo invasion through the luminal epithelium, stromal cell decidualization as well as uterine angiogenesis.

2.3- Results

ERα is Localized to Uterine Stromal Cells During the Decidualization Phase of Pregnancy

ERα protein is present in multiple uterine cellular compartments during early pregnancy. On day 1 of pregnancy, ERα is localized to the luminal and glandular epithelial cells as well as in the stroma (Fig. 2.1 A). Similarly, ERα is present in these same cellular compartments on day 4 of pregnancy, a time when the embryo will attach to the luminal epithelium (Fig. 2.1 B). On day 5 of pregnancy, at the onset of decidualization, ERα is abundantly present in the uterine

stromal cells (Fig. 2.1 C). This spatio-temporal localization of ERα indicates that this protein may play a role in decidualization.

ERα is Essential for Decidualization during Early Pregnancy

To understand the functional role of ERα during decidualization, we created a conditional knockout mouse for the ERα gene. Mice harboring loxp sites flanking the exon 3 of the ERα gene, ERα floxed mice, were crossed with mice expressing cre-recombinase under the endogenous PR promoter. The conditional knockout mice are termed $ER\alpha^{d/d}$ mice and the flox only control mice are $ER\alpha^{f/f}$ mice.

$ER\alpha^{d/d}$ mice exhibit an efficient deletion of ERα in the luminal epithelium, glandular epithelium and stromal compartment of the uterus (Fig. 2.2 B). As expected, these mice are infertile (Table 2.1).

Both $ER\alpha^{d/d}$ and $ER\alpha^{f/f}$ mice were subjected to artificial decidualization protocol and uterine horns are collected 72hrs after administration of a decidual stimulus. $ER\alpha^{f/f}$ mice show a robust decidual response after injection of an oil stimulus into the uterine lumen (Fig. 2.2 C). The left uterine horn was used as an internal unstimulated control. $ER\alpha^{d/d}$ mice fail to form any deciduoma in response to an oil stimulus.

Impaired Decidualization and Angiogenesis in $ER\alpha^{d/d}$ Uteri

$ER\alpha^{d/d}$ mice fail to form a deciduoma after artificial decidual stimulus. We next examined two hallmarks of decidualization and angiogenesis. Alkaline phosphatase activity is an indicator of cellular differentiation and platelet/endothelial cell adhesion molecule 1 (PECAM1) marks endothelial cells indicating the vascular network formation in the decidua. $ER\alpha^{f/f}$ uterine stromal cells show a robust response to the decidual stimulus. The stromal compartment shows a high level of alkaline phosphatase activity indicating that these stromal cells have differentiated

(Fig. 2.3 A). ERα$^{d/d}$ uterine stromal cells however lack alkaline phosphatase activity indicating that uterine stromal cell differentiation is dependent on ERα (Fig. 2.3B).

The second hallmark of decidualization is an up-regulation of PECAM1 indicative of enhanced angiogenesis. Within the uterine compartment of ERα$^{f/f}$ mice there is a high expression of PECAM1. The endothelial cells marked with this antibody have formed a vascular network surrounding the site of stimulation (Fig 2.3 C). In contrast, ERα$^{d/d}$ uterine compartment fails to develop this vascular network surrounding the uterine lumen (Fig. 2.3 D). Collectively, these results indicate that ERα plays an essential role in both stromal cell differentiation and angiogenesis during decidualization.

ERα$^{d/d}$ Uteri Fail to Express PR and Other Biochemical Molecules Essential for Decidualization

To confirm our observation that ERα$^{d/d}$ stromal cells fail to decidualize, we examined the expression of known biochemical molecules which are essential for decidualization. PR plays an essential role in decidualization as uteri from PR knockout mice lack the ability to decidualize in response to an artificial decidual stimulus (40). While ERα$^{f/f}$ uterine sections show prominent PR expression, ERα$^{d/d}$ uterine stromal cells fail to express PR mRNA or protein, indicating compromised deciualization in absence of ERα (Fig. 2.4 B&C).

Prolactin-related protein (PRP) is a well known marker of stromal cell decidualization (41). ERα$^{d/d}$ uterus fails to express PRP mRNA after artificial decidual stimulus. In addition, expression of several molecules critical for decidualization including CCAAT/enhancer binding protein beta (C/EBPβ), bone morphogenetic protein 2 (BMP2), and wingless-related MMTV integration site 4 (WNT4) are down-regulated in the ERα$^{d/d}$ uterus (Fig. 2.4 D) (42-43). It is likely that ERα is a master regulator of this cellular differentiation event.

Stromal ERα Expression is Critical for Decidualization

The ERα$^{d/d}$ uterus has an efficient deletion of ERα in both uterine epithelial cells as well as stromal cells (Fig. 2.2B). The question then arises, is ERα acting directly in the uterine stromal cells to promote decidualization or is ERα acting within the uterine epithelial cells via an indirect mechanism to promote decidualization? To address this question, we employed an *in vitro* decidualization experiment. Primary uterine stromal cells were isolated directly from the mouse uterus and decidualized in culture after administration of E and P (43). Using this method, stromal cells decidualize *in vitro* independent of epithelial cells.

Uterine stromal cells were isolated from ERα$^{f/f}$ and ERα$^{d/d}$ mice and cultured in presence of E and P. As stromal cells decidualize in culture, they take on a fibroblast morphology as seen with ERα$^{f/f}$ stromal cells after 24 and 48 hrs of culture (Fig. 2.5 A&C). ERα$^{d/d}$ stromal cells fail to take on this decidual morphology (Fig. 2.5 B&D). To confirm our observations, we quantified PRP mRNA expression, a marker of decidualization. In contrast to ERα$^{f/f}$ stromal cells, ERα$^{d/d}$ uterine stromal cells fail to express PRP mRNA levels (Fig. 2.5F). Collectively, both morphological and biochemical analyses indicate that ERα in uterine stroma is essential for decidualization.

Microarray Analysis Indicates Regulation of Cell Cycle Genes by ERα

To identify down-stream molecular targets of ERα within uterine stromal cells during decidualization, we employed microarray analysis. ERα$^{f/f}$ and ERα$^{d/d}$ mice were subjected to artificial decidual stimulus and uterine stromal cells were isolated 10 and 24 hrs post-oil stimulus. RNA was isolated from these uterine stromal cells and subjected to microarray analysis using Affymetrix chips. Microarray data were analyzed using Panther Analyses, which indicated a large portion of these aberrantly regulated genes to be involved with the cell cycle (Fig. 2.6). Further analysis using Ingenuity Pathway Analysis indicated that canonical pathways associated

with polo-like kinases, essential molecules for mitosis, are aberrantly regulated in absence of ERα (data not shown).

To determine if mitosis is affected during stromal cell decidualization in absence of ERα, $ERα^{f/f}$ and $ERα^{d/d}$ mice were subjected to artificial decidualization and uterine horns were collected 16 hrs post stimulus. $ERα^{f/f}$ stromal cells express a high level of phospho-histone 3, a marker of mitosis, surrounding the uterine lumen (Fig. 2.7A). $ERα^{d/d}$ stromal cells fail to express this essential mitosis marker during decidualization indicating that uterine stromal cells are unable to enter mitosis in absence of ERα (Fig. 2.7B).

To determine if uterine stromal cells are able to enter the cell cycle in absence of ERα, we analyzed the expression of multiple cell cycle genes in stromal cells of $ERα^{f/f}$ and $ERα^{d/d}$ mice 10 hrs post artificial decidual stimulus. During proliferation, cells enter the G1 phase of the cell cycle from a resting state, G0. Cells continue through the cell cycle from G1 to S phase, DNA replication, to G2 phase and then to mitosis, M phase, for cell replication. The cell cycle is tightly controlled by cyclins, cyclin-dependant kinases (Cdk), and other check-point molecules (44).

The D family of cyclins as well as Cdk4 are known molecules that are essential in the early G1 phase of the cell cycle. Following 10 hrs of decidualization *in vivo*, $ERα^{d/d}$ stromal cells express cyclins D1, D3 and Cdk4 at higher levels than $ERα^{f/f}$ stromal cells (Fig. 2.8A) indicating that these cells have the ability to enter the cell cycle in absence of ERα. However, expression of late G1 molecules, such as cyclin E1, Cdk2, E2F1 and E2F3, is down-regulated during decidualization in absence of ERα (Fig. 2.8B). Other molecules essential for S, G2 and M phases of the cell cycle, cyclin B2 and Cdk6, are also down-regulated during decidualization in absence of ERα (Fig. 2.8C). Collectively, these data indicate that in absence of ERα, uterine stromal cells lack the ability to progress in the cell cycle beyond the G1 phase.

ERα Binding Directly to EREs Facilitates Uterine Stromal Cell Decidualization

To identify direct down-stream targets of ERα, it is essential to know if ERα binds directly to EREs within genomic DNA to regulate molecular targets or if ERα binds to other transcription factors to regulate down-stream targets during decidualization. To understand how ERα elicits a transcriptional response *in vivo*, we subjected non-classical ERα knock-in mice (NERKI) to artificial decidual stimulus. ERα expressed in NERKI mice lacks the ability to bind directly to genomic DNA.

Mice were subjected to artificial decidual stimulus and uterine horns were collected 72 hours after decidual stimulus was administered. Multiple mice of 'control' genotypes were employed including wild-type (wt), homozygous ERα floxed (flox/flox), heterozygous ERα conditional knockouts using PR-cre (cre/+ fl/+) and heterozygous global ERα knockout mice (+/-). All of these control mice responded to the stimulus and developed deciduoma (Fig. 2.9A). However ERα global knockouts (ERαKO-Chambon mouse), ERα conditional knockouts driven by PR-cre (cre/+ fl/fl) and NERKI animals failed to elicit a decidual response. These data conclude that ERα binding directly to EREs within genomic DNA regulates down-stream molecular targets that are essential for uterine stromal cell decidualization.

Hif2α is Regulated by Estrogen during Decidualization and Angiogenesis

We have previously uncovered the essential role that local estrogen plays in the uterus during decidualization and angiogenesis (24). In response to aromatase inhibitors, which prevent E synthesis, Hif2α is down-regulated in uterine stromal cells (Fig. 2.10). These published data indicate that estrogen regulates Hif2α expression during uterine angiogenesis.

Spatio-temporal Localization of Hif2α in the Endometrium During Early Pregnancy

We analysed Hif2α protein expression in pregnant uteri using a Hif2α specific antibody. Protein expression of Hif2α on day 4 morning of pregnancy is quite low (Fig. 2.11A). Nuclear localization of the protein increases in the uterine stromal cells on day 4 midnight presumably in

response to a nidatory surge of estrogen which is critical to initiate embryo attachment to the uterine luminal epithelium (Fig. 2.11B). On day 5 of pregnancy, Hif2α protein remains in the nucleus in a small number of stromal cells immediately surrounding the implanted embryo (Fig. 2.11C). Uterine stromal cell nuclear expression of the Hif2α protein significantly increases on day 6 of pregnancy and continues through day 8 of gestation, a time in which uterine vascularization supports embryonic growth prior to placentation (Fig. 2.11 D-F). The spatio-temporal localization of Hif2α indicates that this protein may play a critical role during early pregnancy.

Hif2α $^{d/d}$ Mice are Infertile, Indicating an Essential Role of Hif2α in the Uterus during Pregnancy

To study the functional role of Hif2α in the uterus during pregnancy, a conditional knockout mouse was created. Transgenic mice with loxp sites flanking exon 2 of the Hif2α gene, Hif2α$^{f/f}$, were crossed with PR-cre mice. The resulting conditional knockout mouse, termed Hif2α$^{d/d}$, showed an efficient knock-down of Hif2α mRNA in uterine stromal cells of (Fig. 2.12).

Hif2α$^{d/d}$ and Hif2α$^{f/f}$ female adult mice were mated with wild-type C57 male mice for a period of 6 months. Hif2α$^{f/f}$ female mice averaged 7.89 pups per liter and 5.4 liters during the 6 month mating period. None of the Hif2α$^{d/d}$ mice produced any pups, indicating they were infertile (Table 2.2). These data conclusively indicate an essential functional role of Hif2α during pregnancy.

Embryos Travel into the Uterus of Hif2α $^{d/d}$ Mice During Pregnancy and Initiate a Decidual Response

To explore the role of Hif2α in the uterus during pregnancy, Hif2α$^{d/d}$ and Hif2α$^{f/f}$ female adult mice were mated with wild-type C57 male mice and euthanized on different days of pregnancy. Prior to euthanasia on day 5 of pregnancy, Chicago blue dye was injected into the tail vein of female mice. Chicago blue dye travels through the vasculature into the uterus to the

places of highest vascularization marking an embryo within the uterus with a blue band. In both Hif2α$^{d/d}$ and Hif2α$^{f/f}$ mice, blue bands can be seen in the uterus on day 5 of pregnancy indicating that embryos are reaching the Hif2α$^{d/d}$ uterus (Fig. 2.13 A&B).

Uteri were next collected from Hif2α$^{d/d}$ and Hif2α$^{f/f}$ mice on day 7 of pregnancy. Gross morphology indicates that the embryos in the uterus of both Hif2α$^{d/d}$ and Hif2α$^{f/f}$ mice have initiated a decidual response. However, deciduas in the Hif2α$^{d/d}$ mothers appear slightly smaller and less filled with blood compared to Hif2α$^{f/f}$ mothers (Fig. 2.13C).

Embryos Attach to the Luminal Epithelium of Hif2α $^{d/d}$ Mice to Initiate a Decidual Response but Fail to Invade Through the Luminal Epithelium

Hif2α$^{d/d}$ and Hif2α$^{f/f}$ mice were mated with wild-type C57 male mice and euthanized on different days of pregnancy. Embryos attach to the luminal epithelium on day 4 midnight of pregnancy (Fig. 2.14 B). By day 5, embryos detach from the luminal epithelium but are still able to initiate a decidual response (Fig. 2.14 D). On day 6 of pregnancy, embryos in Hif2α$^{f/f}$ uterus invade into the uterine stroma and the epithelium is no longer present (Fig. 2.14E). However in Hif2α$^{d/d}$ uteri, embryos are unable to invade through the luminal epithelium, which remains intact (Fig. 2.14F). Cytokeratin 8 immunostaining on day 5 of pregnancy marks the intact luminal epithelium. In contrast, embryos in Hif2α$^{f/f}$ uterus attach to the luminal epithelium and invade through these cells by disrupting epithelial cell tight junctions (Fig. 2.14 G&H).

MUC1 is Down-regulated in the Hif2α $^{d/d}$ Uterine Luminal Epithelium, Indicating a Receptive State

Mucin 1, MUC1, is a cell surface glycoprotein regulated by estrogen in the uterus (46). MUC1 is expressed on day 1 of pregnancy and down-regulated in the luminal epithelium on day 4 of pregnancy indicating a receptive uterus. On days 4 and 5 of pregnancy in Hif2α$^{d/d}$ mice MUC1 is not present is the uterine epithelium (Fig. 2.15 B&D). The lack of MUC1 indicates that the luminal epithelium of Hif2α$^{d/d}$ mice is receptive to the attaching embryo.

Hif2α $^{d/d}$ Uterus has the Ability to Decidualize

To address the role of Hif2α in the process of decidualization in the absence of an implanting embryo, an artificially-induced decidualization experiment was employed. The uteri of Hif2α$^{d/d}$ and Hif2α$^{f/f}$ mice were collected 72 hrs after a stimulus of oil was administered into the uterus. Histological examination clearly indicates a decidual response in both Hif2α$^{d/d}$ and Hif2α$^{f/f}$ uteri (Fig. 2.16 A&B). Wet weight gain data also indicate no difference in the robustness of the response between these two transgenic animals (Fig. 2.16C). However, qPCR analysis indicates a down-regulation of two critical decidual molecules, PRP and Cx43, in the Hif2α$^{d/d}$ deciduoma (Fig. 2.16D). Even though a decidual response is initiated in Hif2α$^{d/d}$ uterus, a lack of essential decidual markers indicates that without Hif2α uterine decidual cells are not fully functional.

Decidualization in Absence of Hif2α is Initiated but Not Complete

We next analyzed the spatial expression of decidual markers during early pregnancy. Two classical indicators of decidualization are an increase in alkaline phosphatase activity and an increase in PRP protein within decidual cells. Alkaline phosphatase activity increases by day 5 of pregnancy, very shortly after decidualization is initiated. However, PRP protein is not increased until day 6 of pregnancy and is a late marker of decidualization. We chose to analyze these two markers to determine when decidualization is affected in the Hif2α$^{d/d}$ uterus.

Alkaline phosphatase activity was assessed in uterine sections from day 7 of pregnancy and in uterine sections after 72 hrs of artificial decidualization. In absence of Hif2α, decidualization is clearly initiated as there is an increase in alkaline phosphatase activity on day 7 of pregnancy (Fig. 2.17D) and 72 hrs after administration of artificial stimulus (Fig. 2.17B). Stromal cells are able to undergo differentiation program in absence of Hif2α.

We next looked at PRP, a late decidual marker. In Hif2α$^{f/f}$ mice, there is an induction of PRP surrounding the site of stimulus 72 hrs after oil administration (Fig. 2.17E). Additionally during pregnancy, Hif2α$^{f/f}$ mice express PRP surrounding the implanted embryo (Fig. 2.17G). However there is a complete lack of PRP expression in the Hif2α$^{d/d}$ uterus either during normal

pregnancy or artificial decidualization (Fig. 2.17 F&H). Clearly the decidualization program is initiated in absence of Hif2α but fails to reach the terminal differentiated state.

Uterine Angiogenesis in Absence of Hif2 is Impaired

Hif2α is known to induce angiogenic responses in a number of tissues. To determine if the Hif2α$^{d/d}$ uterus has the ability to form angiogenic networks during decidualization, we mated Hif2α$^{f/f}$ and Hif2α$^{d/d}$ mice to C57 wild-type males and collected uterine horns on day 7 of pregnancy. Immunohistochemistry was completed on these uterine samples using anti-PECAM1 antibody, a marker of endothelial cells. On day 7 of pregnancy, the Hif2α$^{f/f}$ uterus forms a robust angiogenic network of endothelial cells surrounding the implanted embryo (Fig. 2.18 A). The Hif2α$^{d/d}$ uterus shows a blunted expression of PECAM1 indicating a defect in angiogenesis (Fig. 2.18 B). These data indicate that Hif2α plays an essential role in angiogenesis during decidualization.

2.4- Discussion

According to the New York Department of Health, 7.1 percent of married couples in the United States are infertile. Of the 6 million pregnancies that do occur in the United States, one-third will result in pregnancy loss. Many couples turn to assisted reproductive techniques such as IVF to circumvent infertility issues. Because IVF procedures result in live birth 40-60% of the time, often more than one embryo are transferred to the mother's womb. This may result in multiple live births which can be monetarily, emotionally and physically burdensome to parents. Understanding implantation failure at the molecular level will facilitate the development of treatments and procedures for infertile women. These treatments will increase the success rate of IVF and decrease the chance of unwanted multiple births from this procedure.

By employing the ERα$^{d/d}$ mouse model, we addressed the essential role of ERα in uterine stromal cells. Previous published studies from our laboratory indicate that local E production by uterine stromal cells is essential to facilitate decidualization (24). This local E acts via ERα to promote stromal cell decidualization. Successful decidualization is absolutely critical for embryo

implantation and identification of down-stream targets of ERα is essential to understand cellular events during this process.

In the absence of ERα, uterine stromal cells are unable to differentiate, as shown by the lack of alkaline phosphatase activity and PRP mRNA expression. Furthermore in absence of ERα, uterine stromal cells are unable to express molecules essential for decidualization such as PR, C/EBPβ, BMP2 and WNT4. To identify molecular pathways driven by ERα during decidualization, we employed microarray analysis. This analysis indicates a defect in uterine stromal cell proliferation in absence of ERα. Following up these analyses, molecules essential for cell cycle progression were analyzed in $ER\alpha^{d/d}$ uterine stromal cells post decidual stimulus. $ER\alpha^{d/d}$ uterine stromal cells lack phopho-histone 3 after decidual stimulus. However, early G1 phase molecules, such as cyclin D1 and D3 remain intact. These results indicate that uterine stromal cells lacking ERα fail to fully proliferate but appear to be able to enter the cell cycle.

To further address the role of ERα in stroma proliferation and differentiation, we employed the NERKI mice. These studies indicate that ERα binds directly to genomic DNA to elicit these cellular responses. Further work to identify essential molecular targets of ERα containing ERE sequences within their promoters will be necessary to fully understand how ERα regulates stromal cell decidualization.

In addition to compromised stromal differentiation, $ER\alpha^{d/d}$ mice exhibit a defect in angiogenesis. Angiogensis of the decidua is absolutely necessary to bring essential maternal nutrients to the developing embryo prior to placentation. Our laboratory has identified multiple molecules regulated by E that are critical for angiogenesis during decidualization including hypoxia-inducible factor 2 alpha (Hif2a), angiopoietin 2 (Ang2), and connexin 43 (Cx43) (24-25). These findings warrant further studies on the molecular mechanisms of E signaling to promote angiogensis during early pregnancy.

We have previously identified stromal cell production of estrogen as an essential driver of decidualization and angiogenesis in the uterus. By inhibiting estrogen production with an aromatase inhibitor, we know that this local estrogen regulates key molecules of decidualization,

such as alkaline phosphatase, PRP, and BMP2. Additionally local estrogen regulates other molecules, Hif2α and Ang2, which have been previously published in other systems to regulate angiogenic responses. Therefore, we hypothesized that estrogen regulates Hif2α to mediate angiogenesis in the uterus. To test the function of hif2α we created a conditional knockout mouse model, Hif2α$^{d/d}$ mouse.

Even though Hif2α$^{d/d}$ mice ovulate, they are completely infertile. Surprisingly, we found that embryos attach to Hif2α$^{d/d}$ luminal epithelium but are unable to penetrate through the luminal epithelial cells. However embryos flushed from Hif2α$^{d/d}$ uteri on day 4 and transferred into wild-type mothers will successfully develop to term (data not shown). Therefore the embryos within Hif2α$^{d/d}$ pregnant mice have the ability to penetrate through the luminal epithelial cells. Furthermore Hif2α$^{d/d}$ stromal cells are able to enter the differentiation program as marked by alkaline phosphatase activity following embryo attachment to the luminal epithelial cells. We believe that this is the first animal model to show embryo attachment, and not embryo invasion, as the catalyst for stromal cell decidualization in rodents.

Two possibilities remain to explain why embryos are unable to invade through luminal epithelial cells. Hif2α within luminal epithelial cells could be critical for the disassembly of epithelial cells during implantation. Alternatively Hif2α within stromal cells could regulate some factor X which acts on the epithelial cells to promote epithelial cell disassembly during implantation. While both of these possibilities exist, we favor the second possibility. Our immunohistochemical studies indicate nuclear expression of Hif2α beginning on day 4 midnight when the embryo attaches to the luminal epithelium. Nuclear expression remains in the stromal cells during decidualization. While Hif2α is expressed in the luminal epithelium on day 4 morning, the location of this protein is always cytoplasmic in the epithelial cells. Hif2α must be present in the nucleus to act as a transcription factor. However, to clearly address the role of Hif2α within luminal epithelial cells we are creating a conditional knockout of Hif2α in luminal epithelial cells by employing a mouse expressing cre-recombinase driven by the Wnt7a promoter. Breeding studies with these epithelial cell conditional knockout mice will address the role of Hif2α in uterine luminal epithelial cells during implantation.

It is quite remarkable that Hif2α$^{d/d}$ stromal cells are able to begin decidualization even though the embryo does not come into contact with the stromal cells. Stromal cell expression of alkaline phosphatase activity is unaltered in the Hif2α$^{d/d}$ uterus and uterine wet weight gain is statistically the same as Hif2α$^{f/f}$ mice 72 hours after artificial decidual stimulus. However, Hif2α$^{d/d}$ decidual cells do not express PRP, a classic decidualization marker which is induced beginning on day 7 of pregnancy. This is an indication that Hif2α$^{d/d}$ stromal cells are unable to fully differentiate and may never reach a terminal differentiated status.

We began to study Hif2α function in the uterus with the hypothesis that this transcription factor mediates angiogenesis within the uterus. As marked by PECAM1, we can see that angiogenesis is impaired in Hif2α$^{d/d}$ mice on day 7 of pregnancy. It will be imperative to identify the downstream molecular pathways of Hif2α during stromal cell decidualization. As a first step towards that goal we have isolated mRNA from stromal cells on day 5 morning of pregnancy from Hif2α$^{f/f}$ and Hif2α$^{d/d}$ mice and subjected this mRNA to Affymetrix microarray studies. Analysis of these data will help to identify the downstream molecular targets of Hif2α that are important for uterine stromal cell differentiation and angiogenesis.

2.5- Materials and Methods

Animals and Tissue Collection

All animals were maintained in University of Illinois animal facilities. The health of the animals was monitored by Division of Animal Resources veterinarians. All procedures involving the use of animals were approved by the University IACUC committee. Hif2α floxed mice were obtained from Jax Laboratories. Transgenic female mice were mated to mature C57 wild-type males. The copulatory plug indicates day 1 of pregnancy. Female mice were then subsequently euthanized by carbon dioxide overdose on different days of pregnancy. The uterus was collected and either fixed in 10% buffered formalin for paraffin blocks, frozen or used to isolate stromal cells.

Artificial Decidualization

Decidualization was artificially induced as previously described (25). Mice were ovariectomized and rested for 2 weeks to remove circulating hormones. Animals then were injected with 100 ng estrogen in 0.1ml corn oil s.c. daily for three days and then rested for two days. Following rest, mice were injected with E (10 ng) and P (2 mg) in 0.1ml corn oil for three days. On the third day of E+P injection mice were anesthetized and corn oil was injected into 1 uterine horn. Mice were then injected daily with E+P until mice were euthanized.

Uterine Stromal Cell Isolation

Uterine stromal cells were isolated as previously described (43). Briefly, uteri were removed from mice after euthanasia with carbon dioxide. The uterus was cut open and digested with 6 g/liter dispase (Invitrogen) and 25g/liter pancreatin for 1hr at room temp. The enzymes were quenched with 10% FBS and cells were washed with Hanks balanced salt solution (HBSS). Cells were then incubated in 0.5 g/liter collagenase in HBSS for 1hr at 37^0C. After incubation the tubes were vortexed for 10-12 s until the supernatant became turbid with dispersed cells. The contents of the tube were then passed through a 80-μm gauze filter (Millipore). Cells were resuspended in Dulbeccos modified Eagle's medium-F12 medium (DMEM-F12; with 100 unit/liter penicillin, 0.1 g/liter streptomycin, 1.25 mg/liter fungizone) containing 10% heat-inactivated fetal calf serum and live cells were assessed by trypan blue staining using a hemocytometer.

Immunohistochemistry

Immunohistochemistry was carried out as previously described (25). Paraffin embedded sections were used. Briefly, uterine sections were blocked for 1 hr at room temp with serum. Sections were then incubated with primary antibodies against ERα (Novacastra), PECAM (BD), PR (Dako), phospho-histone 3 (Upstate), PRP (Milipore) or Hif2α (Novus Biologicals) overnight at 4^0C. Sections were then incubated with biotinylated secondary antibodies at room temperature followed by incubation with horseradish peroxidase-conjugated streptavidin (Invitrogen). The sections were stained in AEC solution until optimal signal was obtained.

Alkaline Phosphatase Activity Assay

The protocol for detecting alkaline phosphatase activity was followed as previously published (45). Briefly, frozen uterine sections were incubated in the dark at 37^0C for 60 mins in 2mM α-napthyl phosphate. The uterine alkaline phosphatase activity releases orthophosphate and napthol derivatives from the stubstrate. The napthol derivatives are simultaneously coupled with diazonium salk present in the incubating medium to form a dark dye marking the site of enzyme action. To terminate the enzymatic reaction, the tissue sections were rinsed in water.

Quantitative PCR and RNA Isolation

RNA was isolated from tissues or cells using trizol followed by RNA cleanup kit (Qiagen). RNA was converted to cDNA using a kit (Applied Biosystems). Oligonucleoties specific for genes of interest were developed. QPCR reaction was carried out using SYBR-Green master mix (Applied Biosystems) on either 7000 or 7500 Applied Biosystems Real-time PCR machines.

Microarray Analysis

Mice were subjected to artificial decidualization and uterine stromal cells were isolated 10 and 24 hrs post-stimulus. Messenger RNA was isolated from cells using trizol and purified using a Qiagen kit. Messenger RNA was submitted to the Keck Biotechnology Center at UIUC and hybridization on Affymetrix chips was performed. Data were analyzed using Panther and Ingenuity Pathway Analysis software.

2.6- Figures and Tables

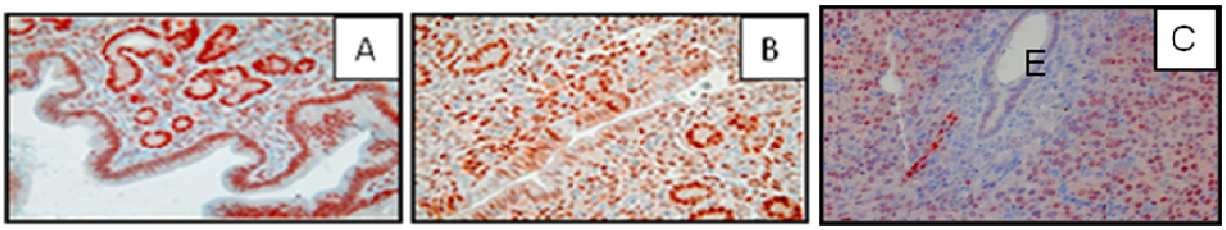


Fig. 2.1: ERα Protein Localization in the Mouse Uterus During Early Pregnancy. Stromal cell localization of the ERα protein is present on day 1 (A) and day 4 (B). ERα expression in the stromal compartment persists on day 5 (C), the beginning time point of stromal cell differentiation. Red indicates ERα protein expression. Blue is hemotoxylin marking the nucleus of all cells.

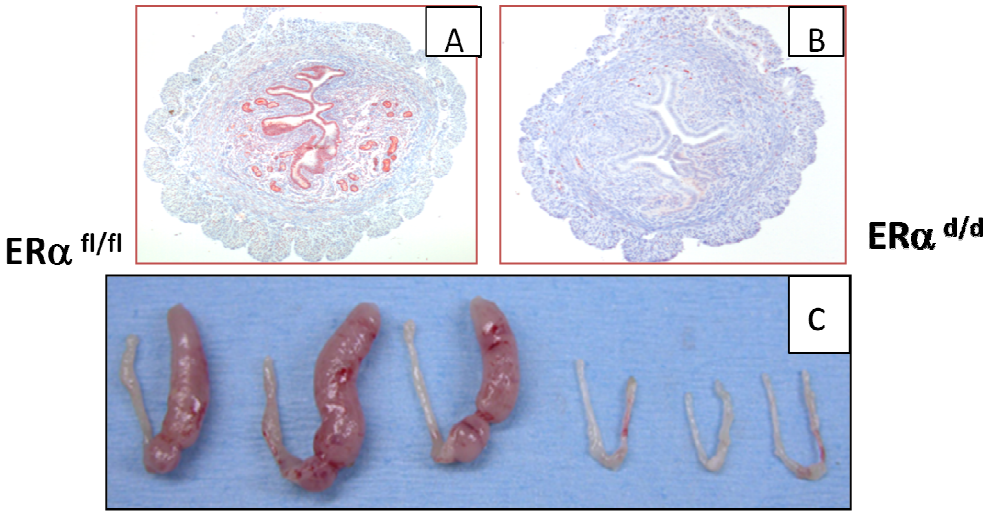


Fig. 2.2: ERα$^{d/d}$ Mice Fail to Display a Decidual Response After Artificial Decidual Stimulus Administration. Uterine cross sections from ERα$^{f/f}$ and ERα$^{d/d}$ mice were subjected to immunohistochemistry using anti-ERα. ERα$^{d/d}$ mice display efficient deletion of ERα (B) when compared to ERα$^{f/f}$ control mice (A). ERα$^{f/f}$ and ERα$^{d/d}$ mice were subjected to an artificial decidual stimulus. Uteri were collected 72 hours after administration of oil stimulus. ERα$^{d/d}$ uteri fail to give a decidual response (C).

Genotype	Number	Number of Liters	Avg. pups/liter	Ovulation ova/ovary (n=5)*
ERαfl/fl	5	14	7.5+/-1.23	18.2+/-.6
ERαd/d	6	0	0	15.7+/-1.7

*Age 5.5 weeks

Table 2.1: ERα$^{d/d}$ Mice are Infertile. ERα$^{d/d}$ and ERα$^{f/f}$ female mice were mated with C57 wild-type male mice for a period of 6 months. ERα$^{f/f}$ female mice averaged 7.5 pups per litter and 2.8 liters per female. ERα$^{d/d}$ female mice are completely infertile as they did not give any pups over the 6 month breeding period. ERα$^{d/d}$ and ERα$^{f/f}$ immature female mice will ovulate similar number of oocytes when stimulated with exogenous gonadotropins. However after ERα$^{d/d}$ female mice reach maturity they will no longer ovulate upon super-ovulation.

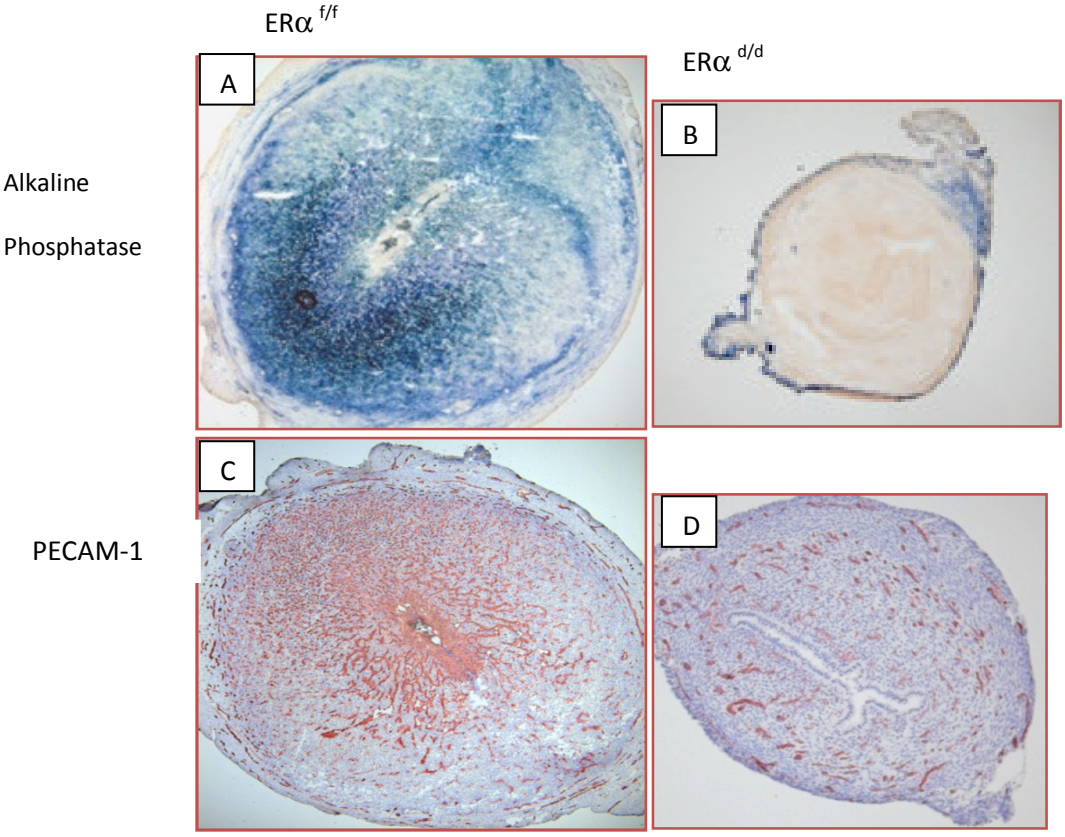


Fig. 2.3: ERα$^{d/d}$ Uteri Fail to Decidualize upon Artificial Stimulation. ERα$^{d/d}$ and ERα$^{f/f}$ uteri were subjected to artificial decidualization and collected 72 hrs after administration of stimulus. Stromal cells in the ERα$^{f/f}$ uterus undergo differentiation following stimulation as marked by alkaline phosphatase (A). ERα$^{d/d}$ uterine stromal cells fail to undergo differentiation and are absent for alkaline phosphatase activity (B). Another hallmark of decidualization is the formation of angiogenic networks surrounding the embryo or site of stimulus. ERα$^{f/f}$ uteri display a vast angiogenic network surrounding the site of stimulus as marked by PECAM-1, an endothelial cell marker (C). Although ERα$^{d/d}$ uteri do have endothelial cells present it is clear that these endothelial cells have not organized into vessels (D). The ERα$^{d/d}$ uterus was unable to decidualize.

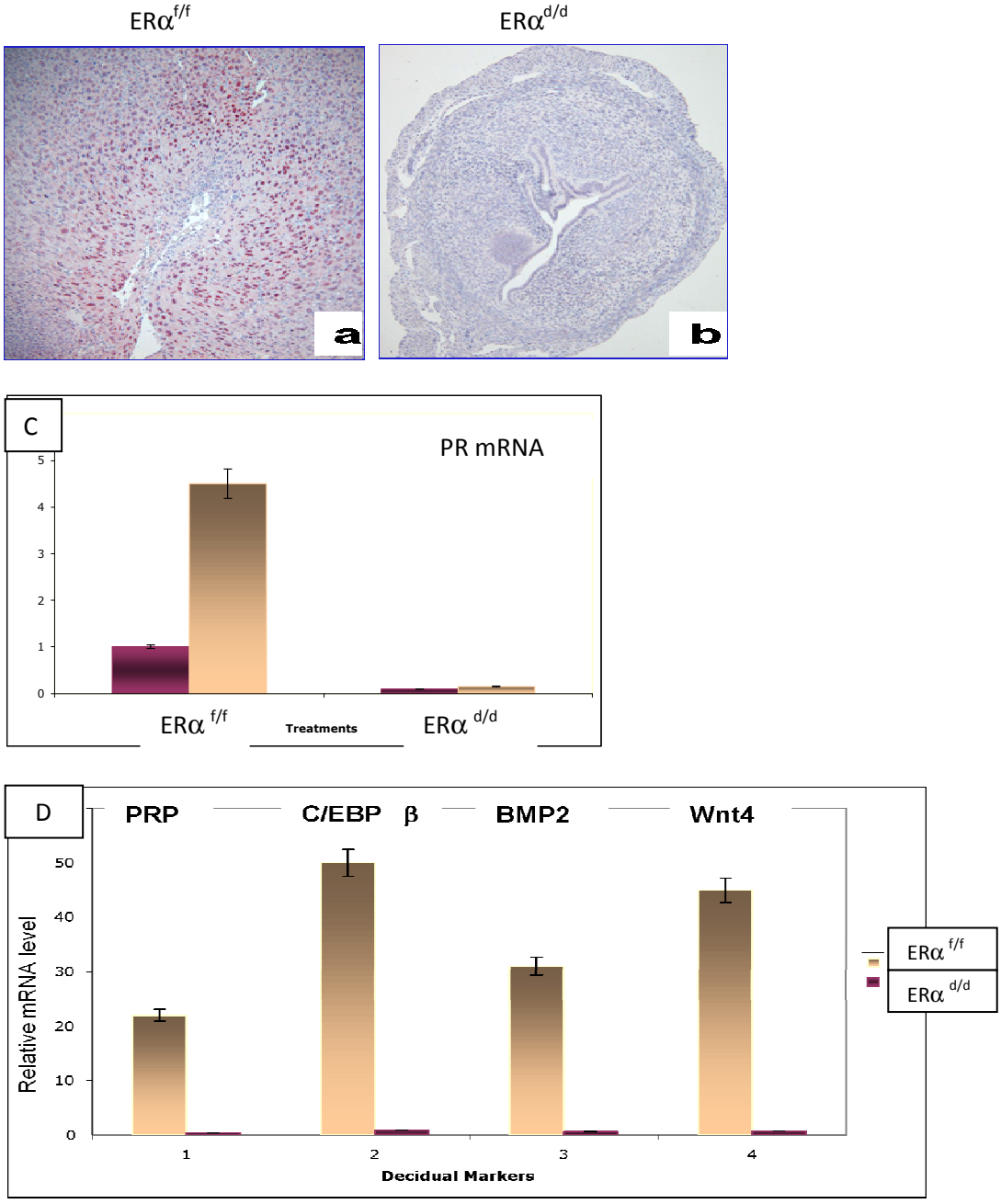


Fig. 2.4: ERα$^{d/d}$ Uteri Fail to Express PR and Other Genes Known to Play an Essential Role in Decidualization. ERα$^{d/d}$ and ERα$^{f/f}$ uteri were subjected to artificial decidualization and collected 72 hrs after administration of stimulus. Immunohistochemistry indicates a high level of PR protein in the decidua of ERα$^{f/f}$ uterus (A). ERα$^{d/d}$ uterus fails to express PR protein following decidual stimulation (B). ERα$^{d/d}$ uterus also fails to express PR mRNA after decidual stimulation (C). Furthermore, ERα$^{d/d}$ uterus also fails to express genes known to play an essential role in decidualization such as PRP, C/EBPβ, BMP2 and WNT4 as measured by quantitative PCR (D).

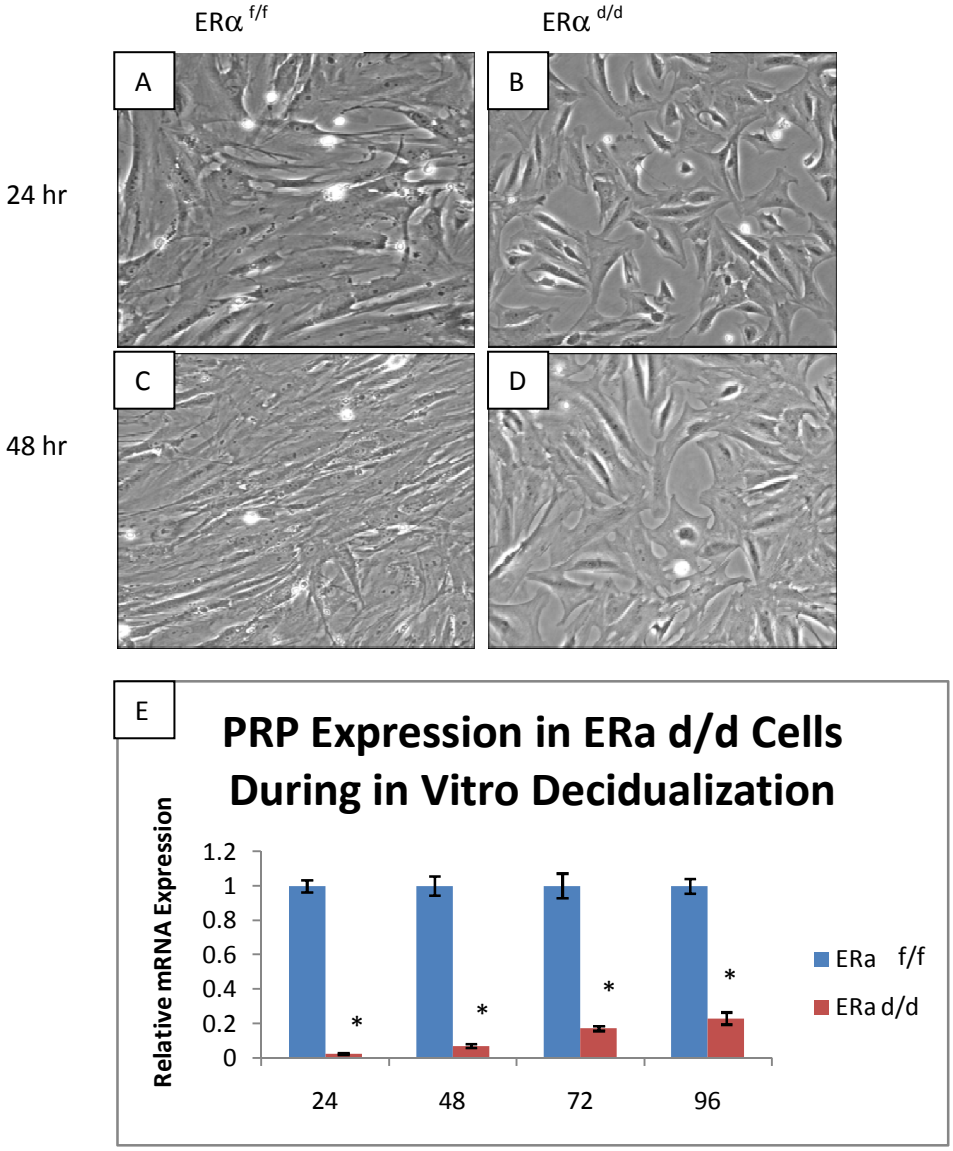


Fig. 2.5: ERα$^{d/d}$ Uterine Stromal Cells Fail to Differentiate in vitro in Absence of Luminal Epithelial Cells. Uterine stromal cells are enzymatically isolated from ERα$^{d/d}$ and ERα$^{f/f}$ female adult mice. ERα$^{d/d}$ and ERα$^{f/f}$ uterine stromal cells are cultured for 24hrs (A&B) and 48hrs (C&D) in the presence of estrogen and progesterone. ERα$^{d/d}$ uterine stromal cells do not express essential levels of PRP and fail to differentiate (E). * indicates a significant difference in gene expression between mice with different genetic backgrounds at a p-value cutoff of 0.05 using a student's t-test.

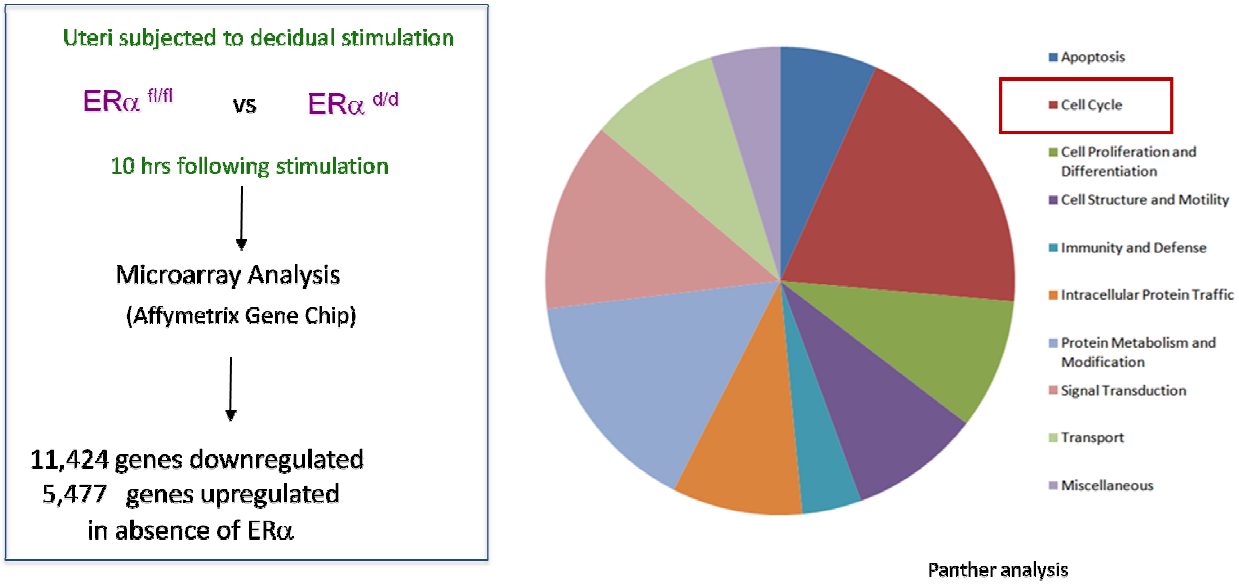


Fig 2.6: Microarray Analysis Indicates that ERα$^{d/d}$ Uterine Stromal Cells Display Aberrant Expression of Multiple Cell Cycle Molecules. ERα$^{d/d}$ and ERα$^{f/f}$ female adult mice are given a decidual stimuls. Mice are euthanized 10hrs post stimulus, uterine stromal cells are isolated, mRNA is isolated from the stromal cells and subjected to Affymetrix microarray analysis. Panther analysis of aberrantly regulated genes suggests that ERα$^{d/d}$ uterine stromal cells aberrantly express a number of cell cycle molecules post stimuls when compared to ERα$^{f/f}$ uterine stromal cells.

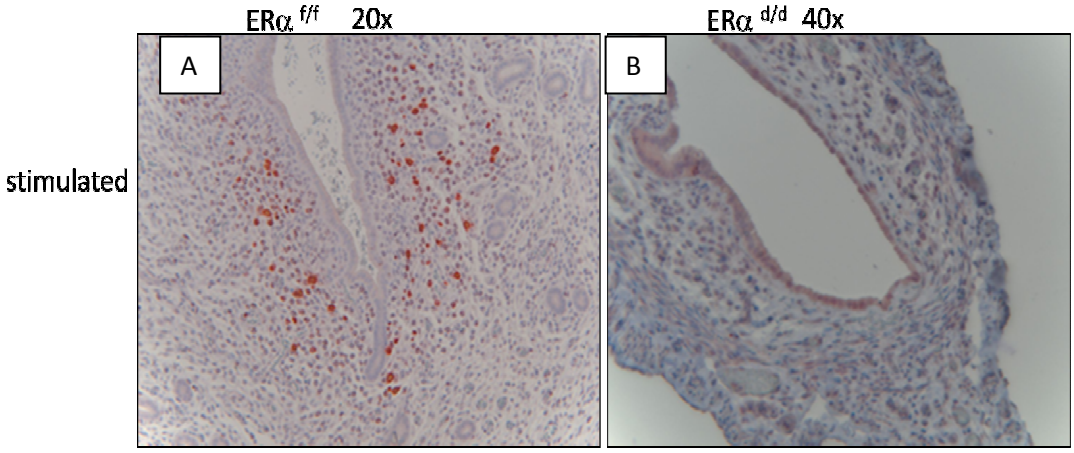


Fig 2.7: ERα$^{d/d}$ Uterine Stromal Cells Fail to Undergo Mitosis Following Artificial Stimulus. ERα$^{d/d}$ and ERα$^{f/f}$ female adult mice are given a decidual stimulus and euthanized 16hrs post stimulus. Uterine cross sections are stained for phospho-Histone 3, a marker of mitosis. ERα$^{f/f}$ uterus completes mitosis following artificial stimulus (A). ERα$^{f/f}$ uterus fails to proliferate following artificial stimulus (B).

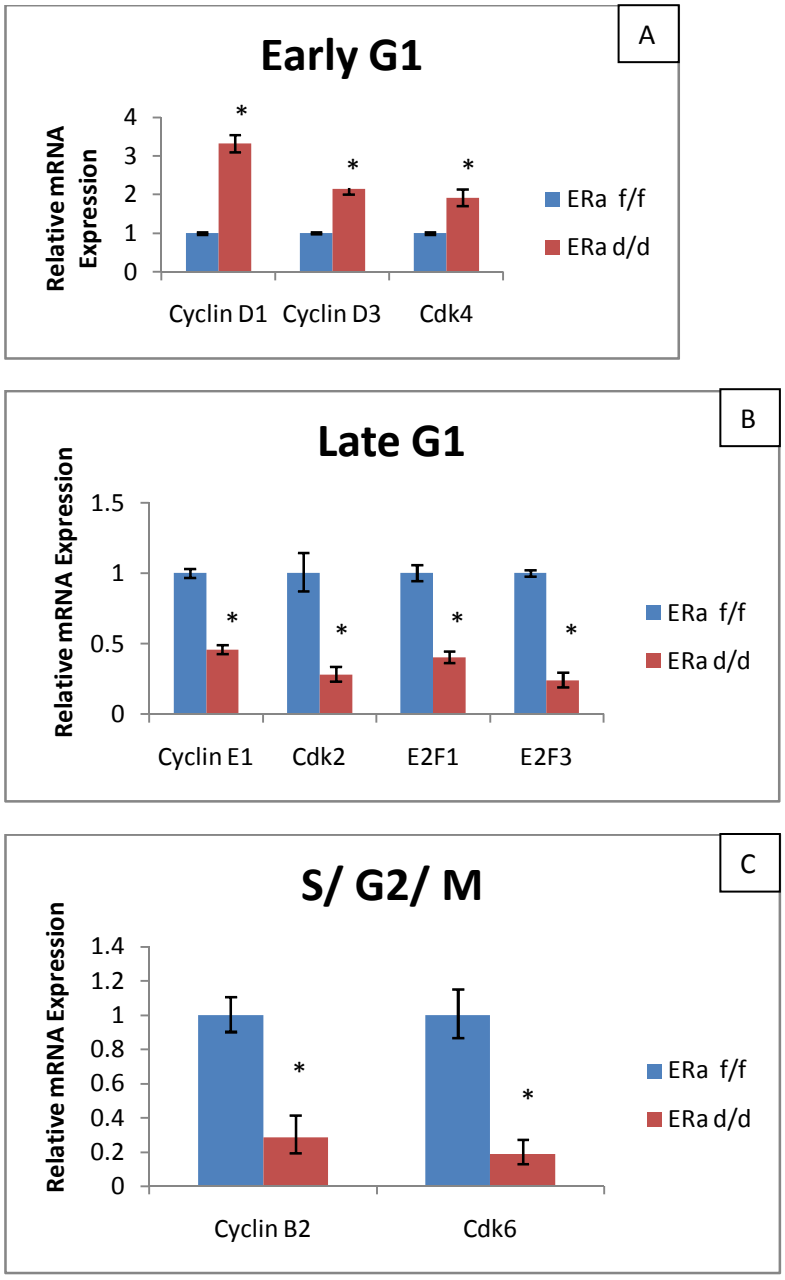


Fig. 2.8: ERα$^{d/d}$ Uterine Stromal Cells Fail to Transition from G1 to S Phase Following Artificial Stimulus. ERα$^{d/d}$ and ERα$^{f/f}$ female adult mice are given a decidual stimulus and euthanized 10hrs post stimulus, stromal cells are isolated and mRNA is extracted. QPCR is employed to measure mRNA levels of cell cycle regulatory genes. ERα$^{d/d}$ uterine stromal cells express adequate levels of early G1 cell cycle genes (A). However ERα$^{d/d}$ uterine stromal cells do not express adequate levels of cell cycle molecules reqired for late G1, S, G2 or M phase (B&C). * indicates a significant difference in gene expression between mice with different genetic backgrounds at a p-value cutoff of 0.05 using a student's t-test.

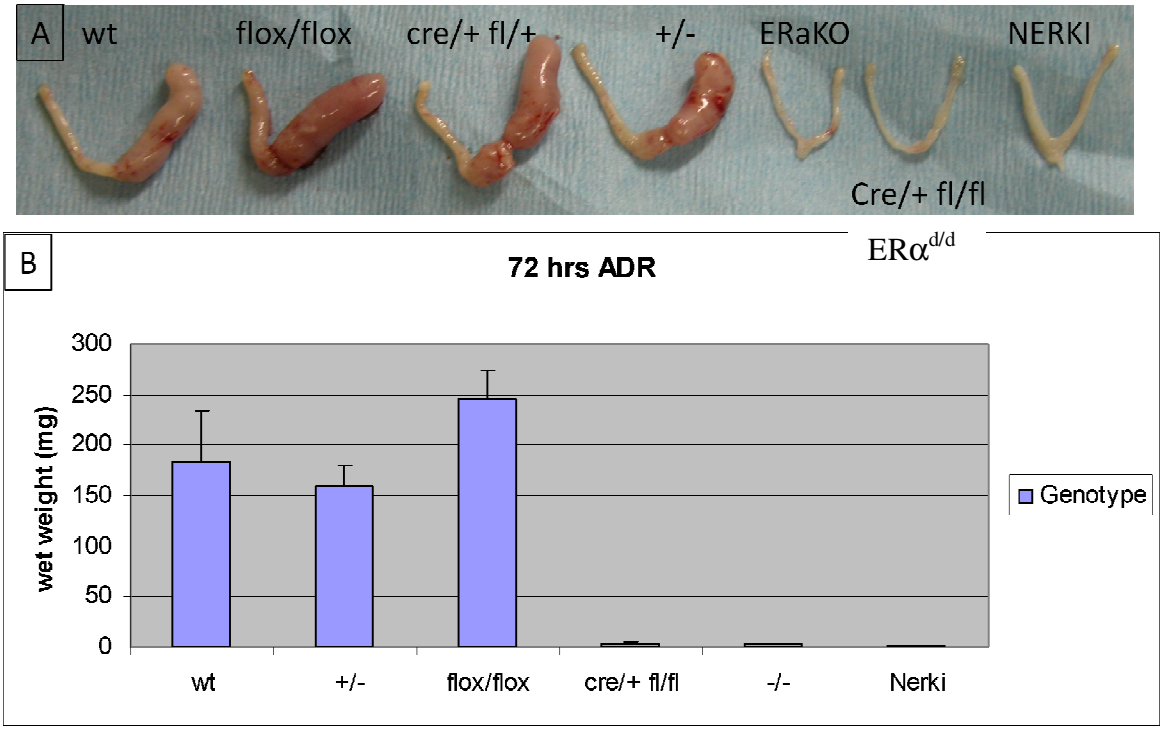


Fig. 2.9: Decidualization Requires ERα to Bind to EREs for Transcription of Downstream Genes. Mice are subjected to artificial decidualization and euthanized 72hrs post stimulation. Mice with intact ERα are able to illicit a decidual response. Mice with ERα knocked out in every cell, ERαKO, or specifically in the uterus, ERα$^{d/d}$, are unable to form deciduomas. Furthermore mice with ERα unable to bind to EREs in the genome, NERKI, are also unable to form deciduomas (A). Uterine wet weight gain for each mouse genotype are depicted in B. Clearly ERα transcription via EREs is required for decidualization.

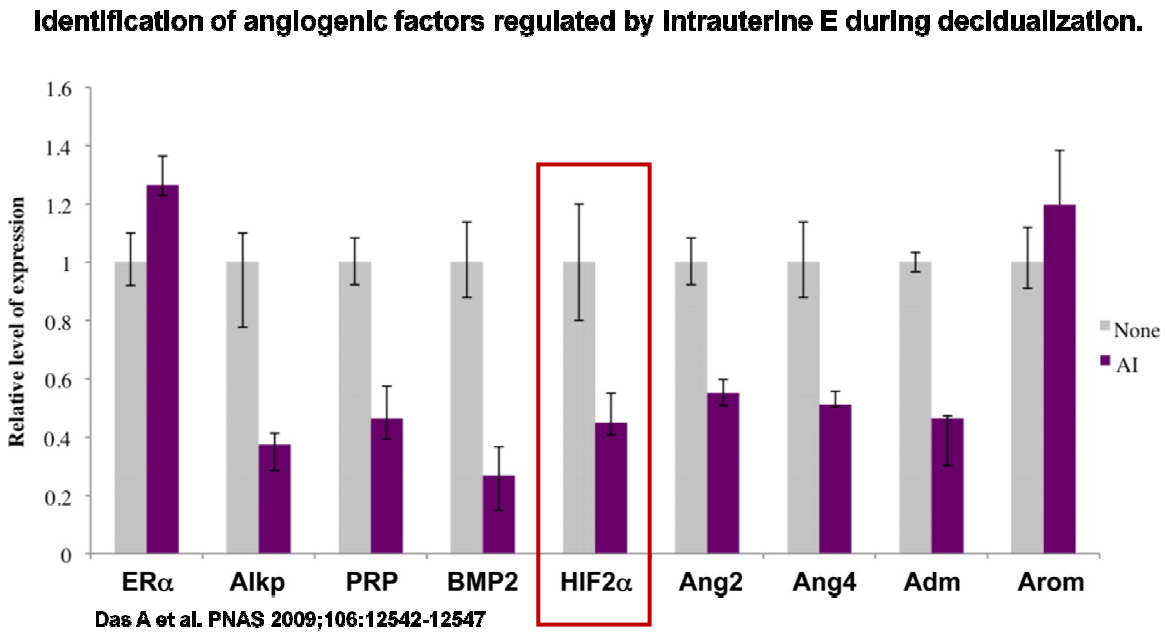


Fig. 2.10: Hif2α is Down-regulated in Presence of Aromatase Inhibitor during *In Vitro* Decidualization. Identification of angiogenic factors regulated by intrauterine E during decidualization. Stromal cells isolated from uteri of d 4 pregnant mice were subjected to *in vitro* decidualization in the presence or absence of letrozole for 72 h. RNA was prepared from these cells and subjected to real-time PCR analysis using gene-specific primers.

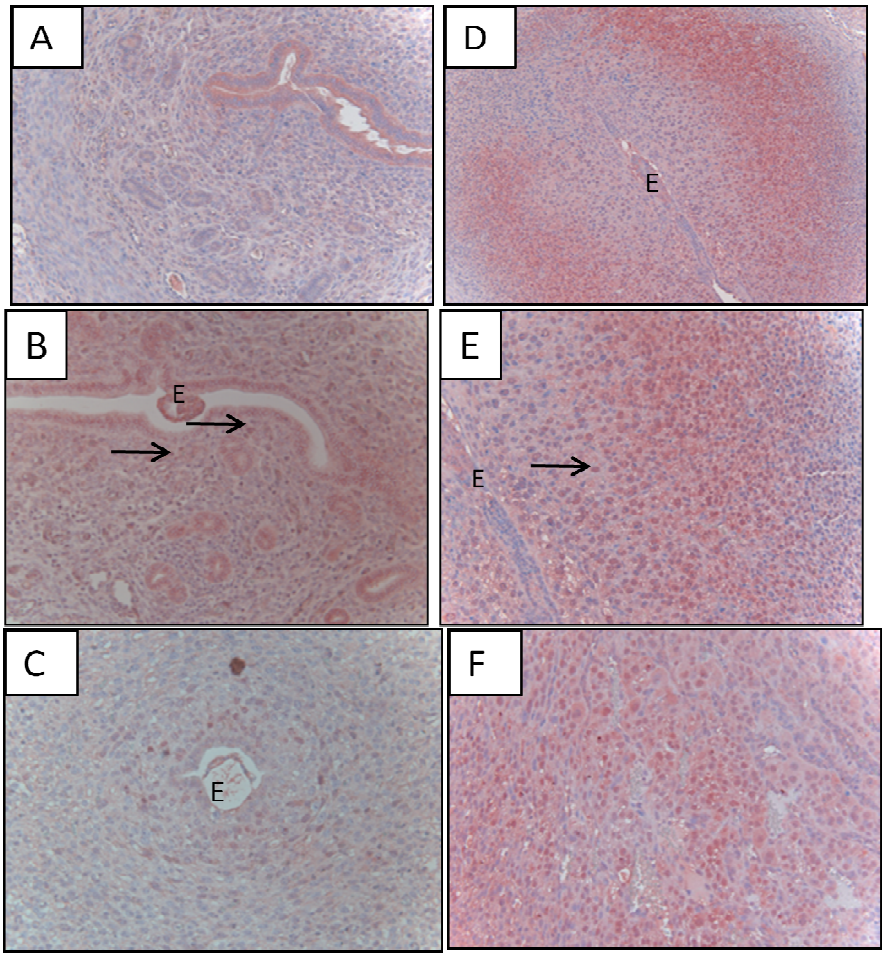


Fig. 2.11: Hif2α Protein Localization in the Mouse Uterus During Early Pregnancy. Expression of Hif2α remains low on day 4 morning of pregnancy (A) However nuclear expression of Hif2α increases by day 4 midnight (B) in uterine stromal cells. On day 5 of pregnancy nuclear expression only remains in a small number of stromal cells immediately surrounding the implanted embryo (C). Protein expression greatly increases on day 6 of pregnancy in differentiated and undifferentiated stromal cells (E). Protein expression remains high through day 8 of pregnancy and is localized to uterine stromal cells surrounding uterine vasculature (F). Black arrows indicate nuclear location of Hif2α.

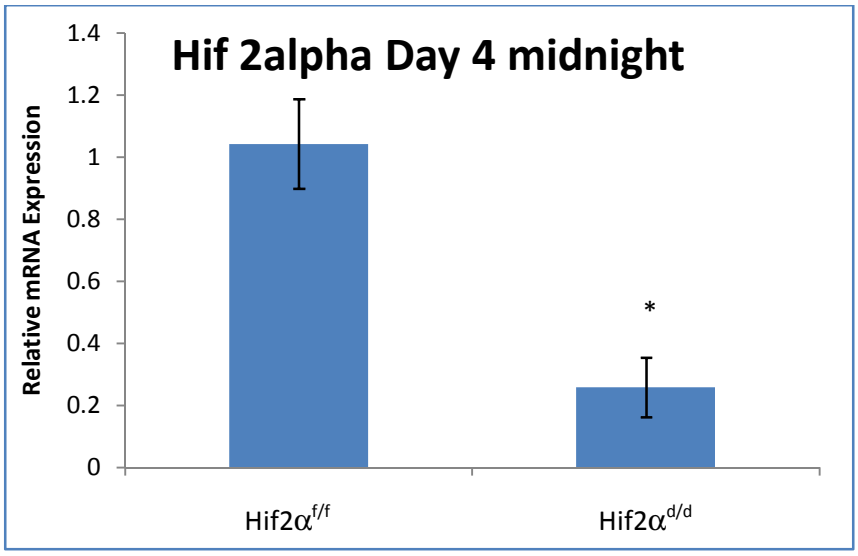
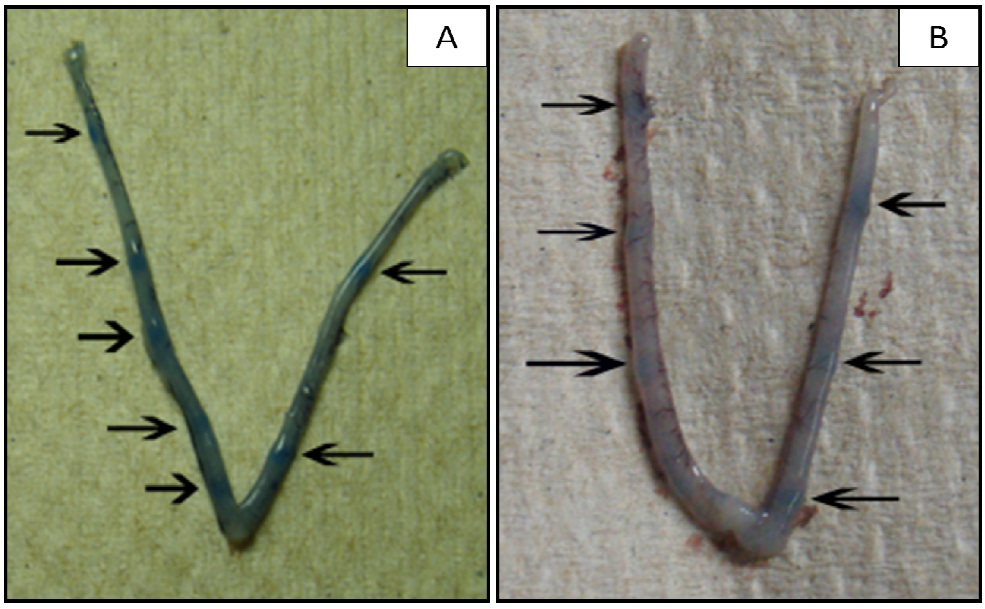


Fig. 2.12: Hif2α$^{d/d}$ Mice have Efficient Knockdown of Hif2α. Hif2α$^{d/d}$ and Hif2α$^{f/f}$ female mice are mated with C57 wild-type male mice and uterine stromal cells are collected on day 4 at midnight and mRNA is isolated. Quantitative PCR indicates an 80% reduction of Hif2α in uterine stromal cells of Hif2α$^{d/d}$ female mice compared to Hif2α$^{f/f}$ control. * indicates a significant difference in Hif2α gene expression between Hif2α$^{f/f}$ mice and Hif2α$^{d/d}$ mice using a p-value cutoff of 0.05. Student's t-test was used to determine statistical significance.

	number of females breeding	number of litters	pups per litter
Hif 2 alpha F/F	5	27	7.89 +/- 0.45
Hif 2 alpha D/D	7	0	0

Table 2.2: Hif2α$^{d/d}$ Mice are Infertile. Hif2α$^{d/d}$ and Hif2α$^{f/f}$ female mice are mated with C57 wild-type male mice for a period of 6 months. Hif2α$^{f/f}$ female mice averaged 7.9 pups per litter and 5.4 liters per female. Hif2α$^{d/d}$ female mice are completely infertile as they did not give any pups over the 6 month breeding period.



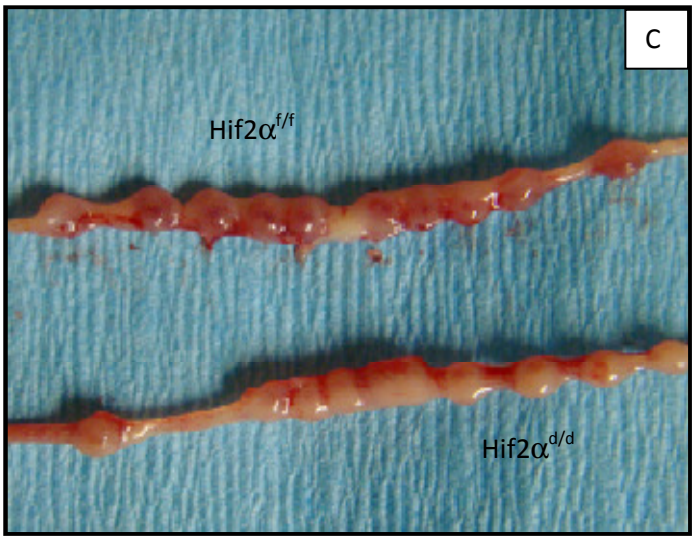


Fig. 2.13: Hif2α$^{d/d}$ Uterus Has the Ability to Vascularize and Decidualize During the Early Stages of Pregnancy. Blue dye is injected into the tail vein of anesthetized mice on day 5 morning of pregnancy. Black arrows point to blue dye indicating embryos in the uterus of Hif2α$^{f/f}$ (A) and Hif2α$^{d/d}$ (B) female mice. Mice are euthanized on day 7 of pregnancy and the uterus is removed. Hif2α$^{f/f}$ and Hif2α$^{d/d}$ uteri appear to decidualize during pregnancy (C).

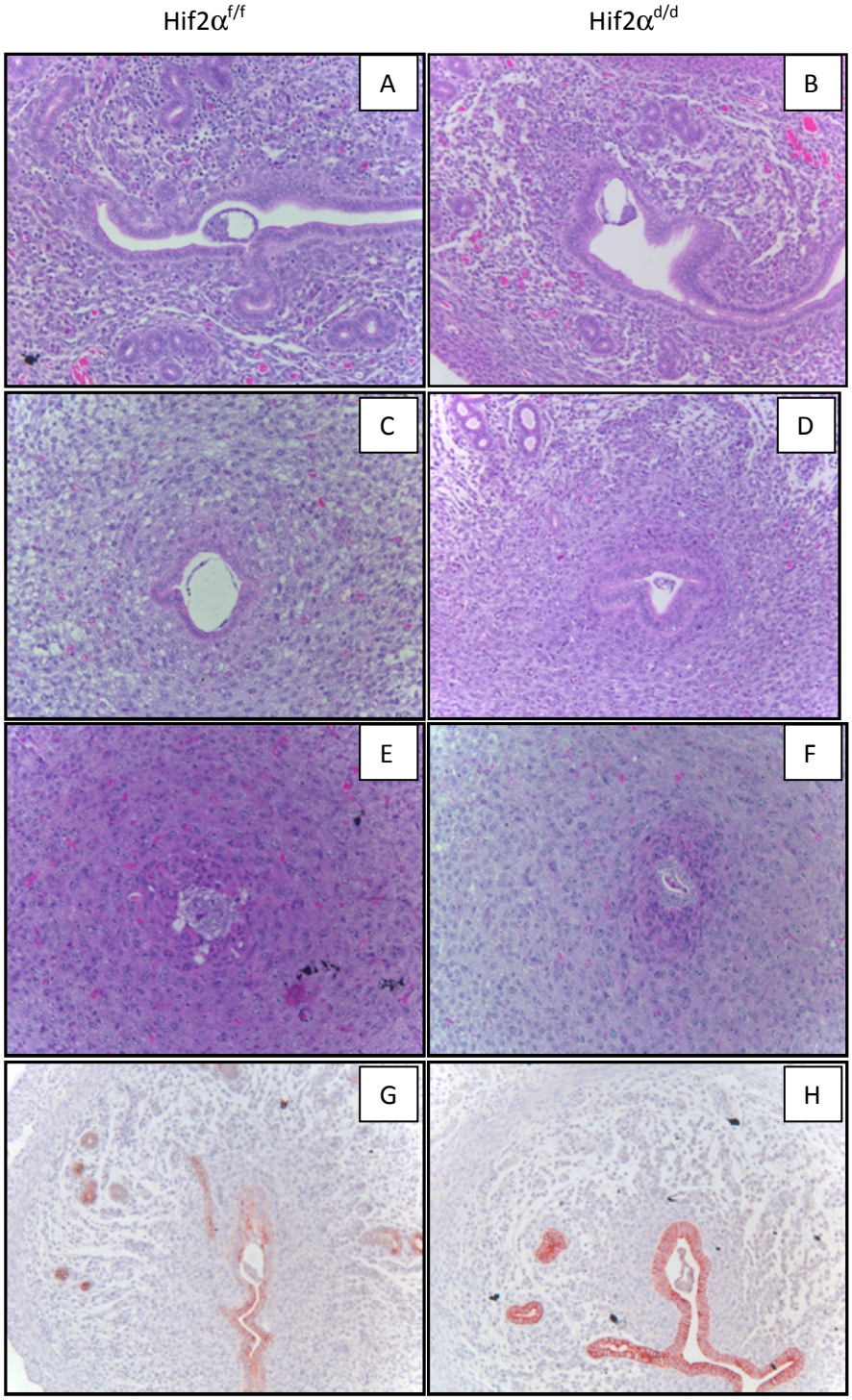


Fig. 2.14: Embryos Fail to Invade Through the Luminal Epithelium in the Hif2α$^{d/d}$ Uterus During Pregnancy. Hif2α$^{f/f}$ and Hif2α$^{d/d}$ female mice are mated to C57 wild-type male mice and euthanized on days 4, 5 and 6 of pregnancy. Embryos attach to the luminal epithelium of both Hif2α$^{f/f}$ (A) and Hif2α$^{d/d}$ (B) uteri on day 4 midnight of pregnancy. The embryo tightly adheres to the luminal epithelium of Hif2α$^{f/f}$ (C) mice but detaches from the luminal epithelium of Hif2α$^{d/d}$ (D) female mice on day 5 morning of pregnancy. Embryos invade through the luminal epithelium of Hif2α$^{f/f}$ mice by day 6 morning (E). Embryos are unable to invade through the luminal epithelium of Hif2α$^{d/d}$ female mice, die and become resorpbed. Cytokeratin 8 marks the luminal epithelial cells in the Hif2α$^{f/f}$ uterus after the embryo invades on day 5 morning of pregnancy. The luminal epithelial cells of the Hif2α$^{d/d}$ remain intact on day 5 morning of pregnancy.

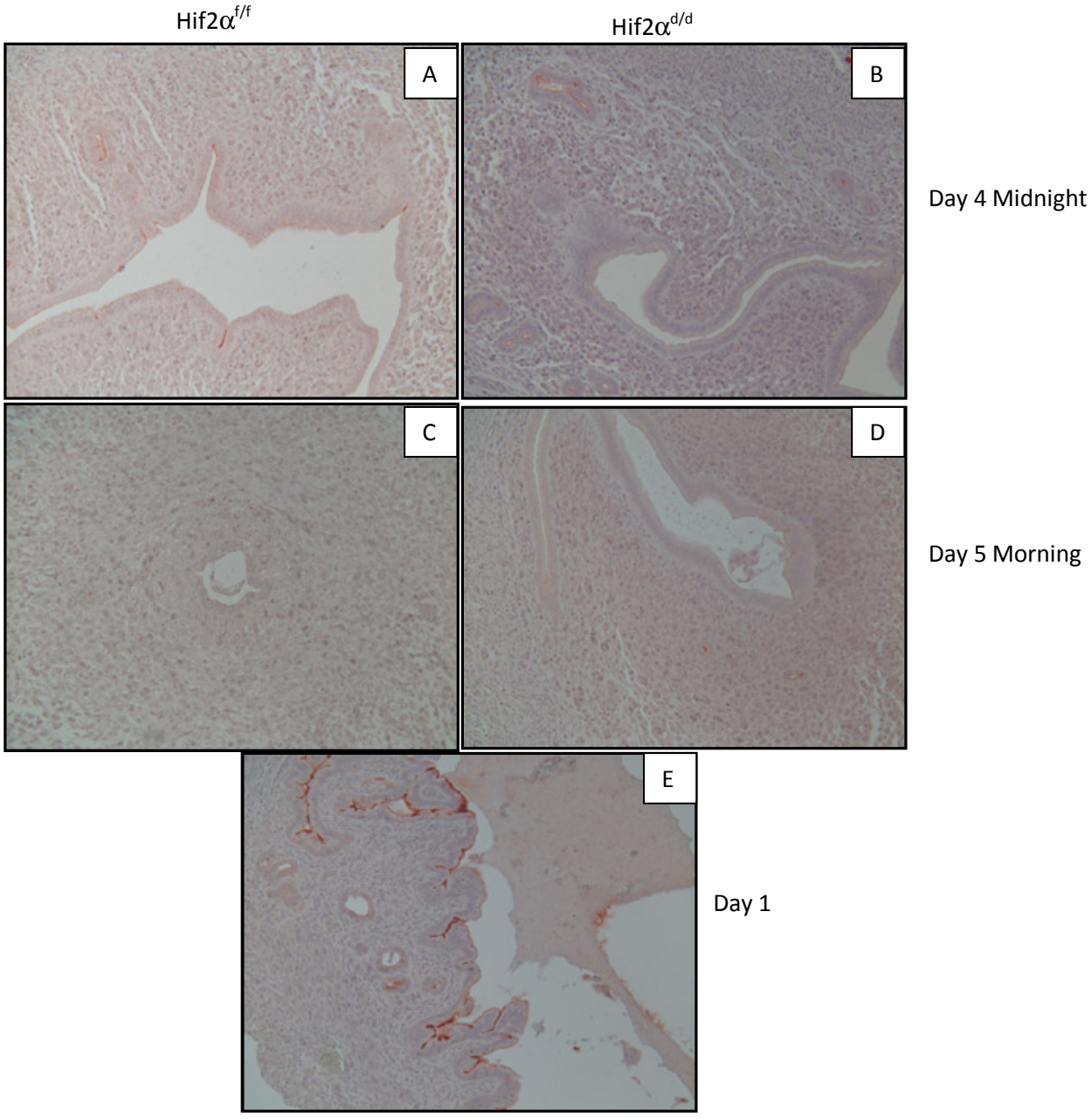


Fig. 2.15: Hif2α$^{d/d}$ Uterine Luminal Epithelium is in a Receptive State During Days 4 and 5 of Pregnancy. Hif2α$^{f/f}$ and Hif2α$^{d/d}$ female mice are mated to C57 wild-type male mice and euthanized on days 4 and 5 pregnancy. The uterine luminal epithelium of Hif2α$^{f/f}$ (A&C) and Hif2α$^{d/d}$ (B&D) mice on day 4 midnight and day 5 morning of pregnancy fails to express MUC1 indicating a receptive state. A uterine cross section of day 1 of pregnancy provides a positive control for MUC1 immunohistochemistry (E).

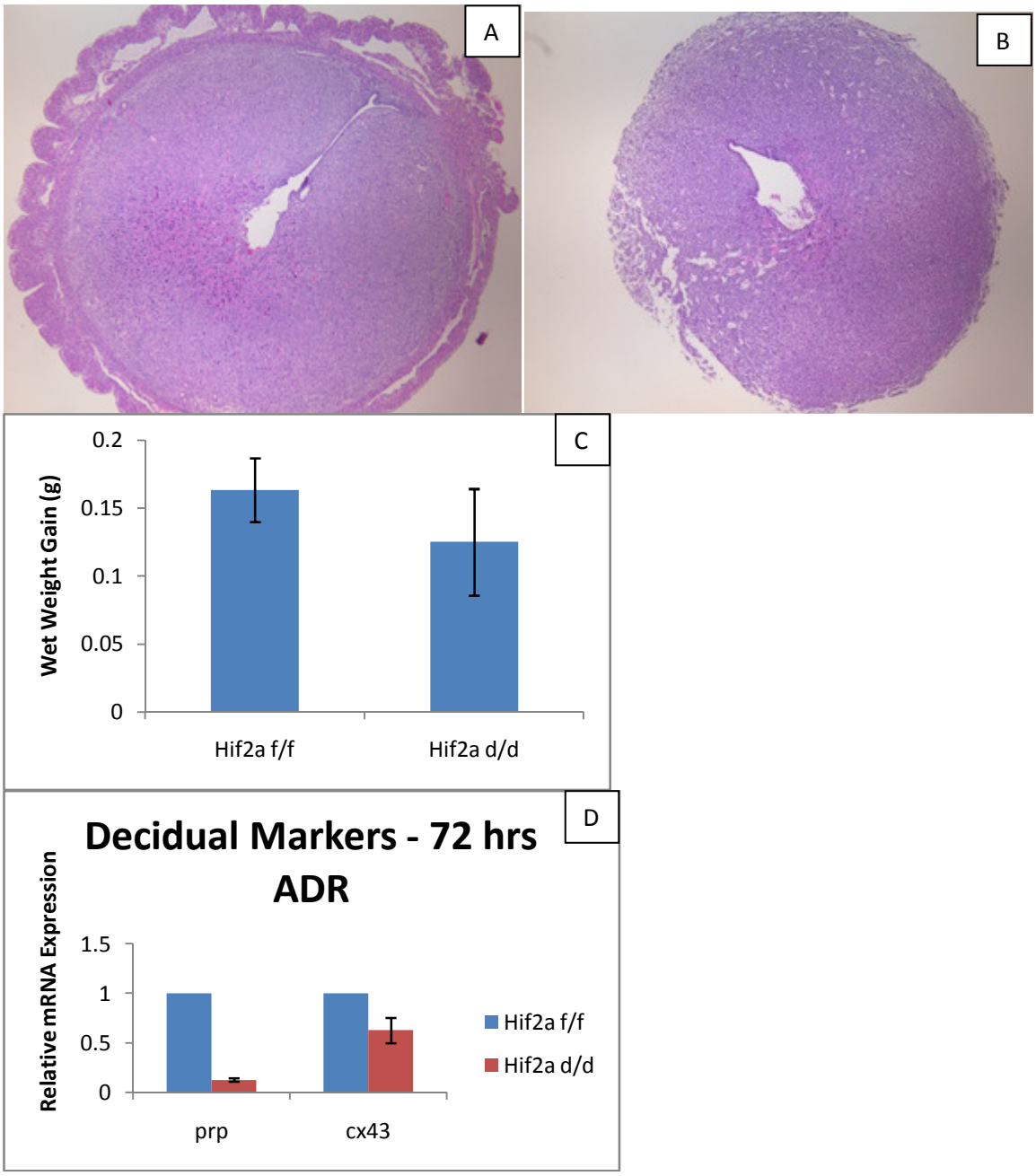


Fig. 2.16: Hif2α$^{d/d}$ Uteri Gain Wet Weight after Artifical Decidual Stimulus but Fail to Express Essential Decidual Genes. Hif2α$^{f/f}$ and Hif2α$^{d/d}$ female mice are subjected to artificial decidual stimulus and euthanized 72hrs post stimulation. Hemotoxylin and Eosin staining indicate decidual response in both Hif2α$^{f/f}$ (A) and Hif2α$^{d/d}$ (B) mice. Uterine wet weight gain does not statistically differ (p= .44) between Hif2α$^{f/f}$ and Hif2α$^{d/d}$ mice after decidual stimulus (C). Despite wet weight gain Hif2α$^{d/d}$ uterine stromal cells fail to express essential levels of PRP and Cx43 mRNA, two well known markers of decidualization (D).

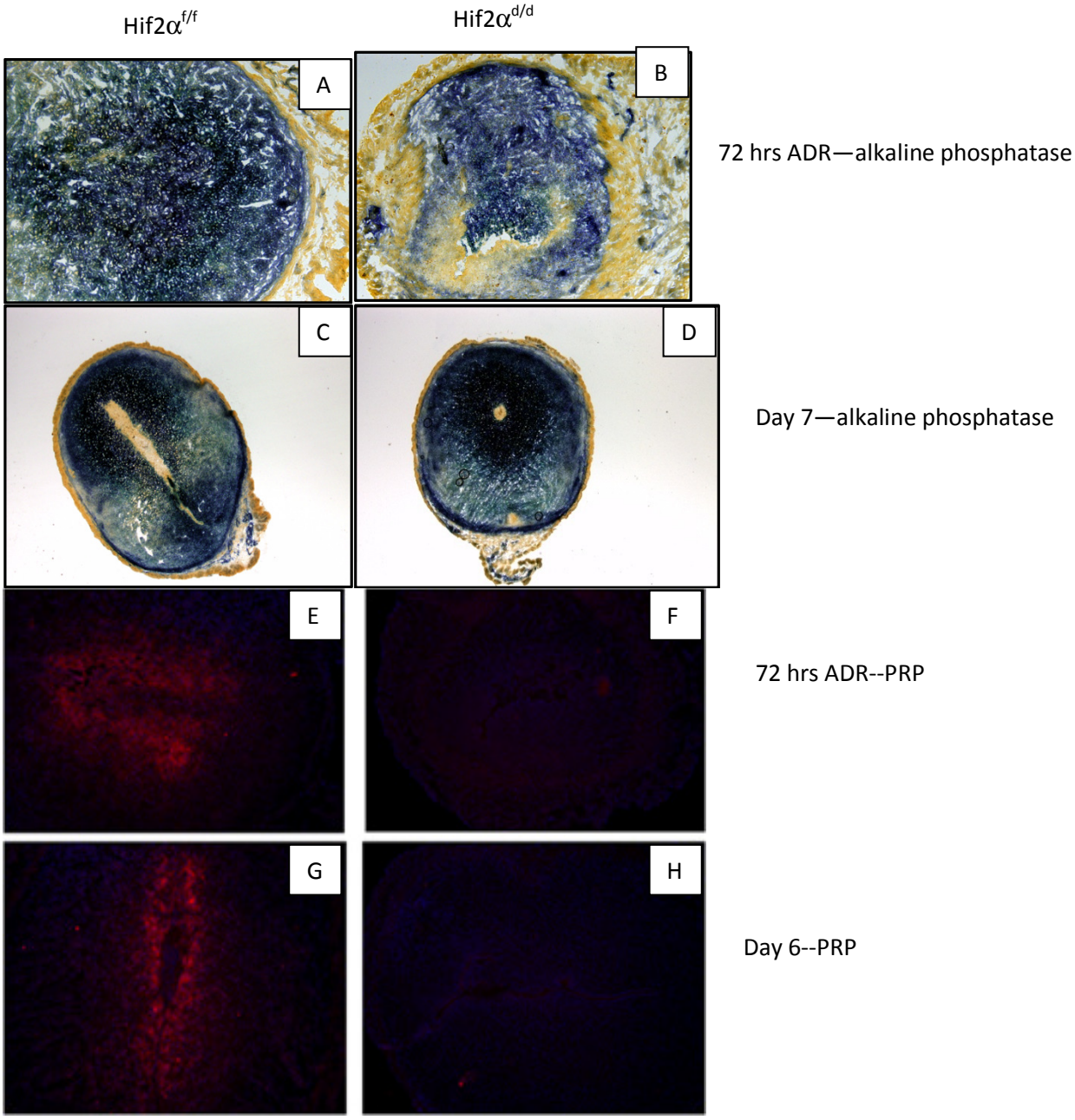


Fig. 2.17: Hif2α$^{d/d}$ Uteri Fail to Fully and Functionally Decidualize. Hif2α$^{f/f}$ and Hif2α$^{d/d}$ female mice are subjected to artificial decidual stimulus and euthanized 72hrs post stimulation. Hif2α$^{f/f}$ and Hif2α$^{d/d}$ female mice are mated to C57 wild-type mice and euthanized on days 6 and 7 of pregnancy. Hif2α$^{f/f}$ uterus displays alkaline phosphatase activity 72 hrs post artificial stimulus (A) and on Day 7 of pregnancy (C) indicating that uterine stromal cells have begun to differentiate into decidual cells. Hif2α$^{d/d}$ uterus also displays alkaline phosphatase activity 72 hrs post artificial stimulus (E) and on Day 6 of pregnancy (G) indicating that uterine stromal cells have fully differentiated into decidual cells. Hif2α$^{d/d}$ uterus fails to expresses PRP protein 72 hrs post artificial stimulus (F) and on Day 6 of pregnancy (H) indicating that Hif2α$^{d/d}$ uterine stromal cells do not have the ability to fully differentiate into decidual cells.

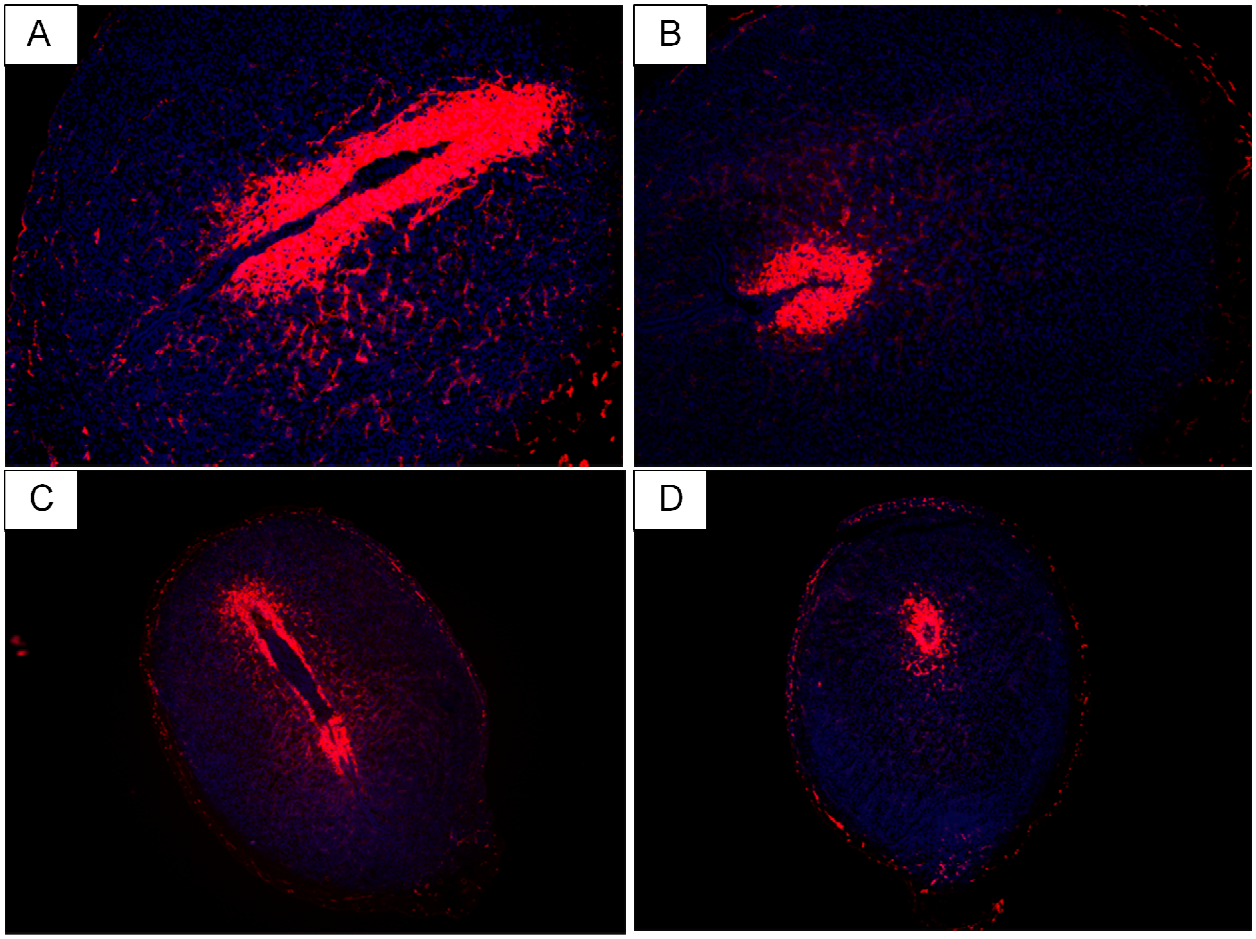


Fig. 2.18: Hif2α$^{d/d}$ within the Uterine Stromal Cells is Essential for Angiogenesis during Decidualization. Hif2α$^{f/f}$ and Hif2α$^{d/d}$ female mice are mated to C57 wild-type mice and euthanized on day 7 of pregnancy. Hif2α$^{f/f}$ uterus expresses a high level of PECAM1 staining marking endothelial cells (A 10x & B 4x). These endothelial cells form a vascular network surrounding the implanted embryo. Hif2α$^{d/d}$ uterus shows a decrease in PECAM1 staining compared to the Hif2α$^{f/f}$ uterus (B 10x & D 4x). Hif2α$^{d/d}$ uterus fails to form vascular networks surrounding the implanted embryo.

Part II: The Role of Estrogen Receptor Alpha in the Ovary During Ovarian Tumorigenesis

CHAPTER 3:Literature Review - Part II

3.1 Ovarian Cancer

Approximately 14,000 women died from ovarian cancer in 2010 in the United States, making ovarian cancer one of the top five types of cancer to cause deaths in women (47). The risk of ovarian cancer increases exponentially with age but plateaus between age 50 to 55. A global perspective on cancer statistics indicates there is not much difference in ovarian cancer risk between developed and non-developed nations. There is a cumulative life-time risk of 1% for women to be diagnosed in developed nations, whereas women in developing nations experience a 0.5% life time risk (48).

Several factors contribute to the risk associated with ovarian cancer. Women who bare children experience a 40% reduction in ovarian cancer risk (49). This could be associated with the reduction in lifetime ovulation events. Women who take oral contraceptives also experience a decreased risk of ovarian cancer. A meta-analysis of 45 studies concluded that for each 5 years of oral contraceptive use a woman reduces her risk of ovarian cancer by 20% (50). This reduction in ovarian cancer risk is thought to be associated with increased periods of anovulation. Some investigators believe that epithelial ovarian cancer cells are derived from germinal inclusion cysts which are thought to form after ovulation by entrapping part of the epithelium below the healing surface of the ovary. However, there is not concrete evidence that the prevalence of germinal inclusion cysts correlates to oral contraceptive use (51). The protective aspect of pregnancy and oral contraception use remains a mystery.

About 15% of ovarian cancer patients report a positive family history of the disease. The discovery of mutations within the breast cancer 1, early onset (BRCA1) gene and breast cancer 2, early onset (BRCA2) gene has been directly correlated with familial history of breast cancer. These are tumor suppressor genes and genetic mutations have been shown to increase a woman's chance of breast cancer by 9- to 36-fold. It was later found that women with these genetic mutations also have a 6- to 61-fold increase risk for ovarian cancer (52). Today, screening for BRCA1/2 mutations allows women to elect to have surgeries such as oophorectomy to reduce the chance of ovarian cancer.

Mutations in tumor suppressor genes are frequently seen in a variety of tumor cases. Mutations in the p53 gene are common among invasive epithelial ovarian tumors. A mutation in this gene is thought to impair the ability of p53 to recognize damaged DNA preventing induction of the normal apoptotic pathway for self-destruction. Studies have indicated that p53 function is lost in 15% of early-stage carcinomas and 50% of late-stage carcinomas, indicating that this mutation may be a late event in tumorigenesis resulting in highly aggressive cancers (53).

Women diagnosed with ovarian cancer have a very low 5-year survival rate. The majority of women are diagnosed with this disease after metastasis to the peritoneum. Women with ovarian tumors usually experience vague clinical symptoms such as bloating or problems with digestion. Detecting ovarian cancer is difficult for clinicians as there are no reliable diagnostic tools. Clinicians have used CA125, an epithelial antigen secreted into the blood, for the past 25 years to diagnosis ovarian cancers. However, the normal serum level of CA125 varies dramatically from patient to patient and often results in false-positive diagnosis of ovarian cancers (54). Undoubtedly, there is a great need to discover dependable diagnostic markers for early stage ovarian cancers.

3.2 Types of Epithelial Ovarian Cancer

Over 90% of the ovarian cancer cases are epithelial cell in nature. Germ cell and sex-cord stromal tumors make up the rest of ovarian cancers. Malignant germ cell tumors are quite rare and are most common in adolescents (55). Malignant sex-cord tumors, which account for about 5% of cancer cases, are more closely related to epithelial ovarian tumors as they are more commonly seen in older women (56). As the vast majority of ovarian cancers are derived from epithelial cells, the different histological types of epithelial ovarian cancer will be reviewed briefly.

Epithelial ovarian cancers are characterized into four main types based on their histology. The most frequent type is serous ovarian cancer accounting for approximately 50%-70% of all epithelial ovarian cancers. Serous epithelial cancer cells resemble the epithelial cells of the Fallopian tube. Based on genetic profiling serous cancers have been separated into two

types, high and low grade. The high grade serous cancers are far more aggressive than the low grade and are characterized by p53 mutations (57).

Endometrioid epithelial ovarian cancer accounts for 15-20% of epithelial ovarian cancer cases and histologically resembles the endometrium. Mutations of CTNNB1, the gene encoding β-catenin, seem to be unique to these types of cancers (58).

Mucinous epithelial ovarian cancer is less frequently diagnosed and accounts for approximately 5-10% of all epithelial ovarian cancers (59). These tumors secrete mucin. The epithelial cancer cells are thought to resemble that of the endocervix.

Accounting for less than 5% of all epithelial ovarian cancers are clear cell tumors and mixed tumors (60). Clear cell tumors have epithelial cells that are thought to resemble that of the gestational endometrium. Mixed tumors are those which develop characteristics of two or more of the other defined histotypes of epithelial ovarian cancer and are extremely rare.

3.3 Transgenic Mouse Models of Epithelial Ovarian Cancer

A lack of sufficient animal models to study the etiology of epithelial ovarian cancer has hindered the development of better diagnostic tools and treatments for epithelial ovarian cancer. Animal models that manifest a pathophysiology similar to humans can be used to better understand specific diseases. Through the use of transgenic mouse models we can identify molecular players that drive a specific pathophysiology. A brief overview of transgenic mouse models that have previously been generated to study epithelial ovarian cancer is outlined below.

Orsulic et al. was perhaps the first to develop a transgenic mouse model for ovarian epithelial cancer by using transgenic animals that expressed the TVA virus receptor driven by the keratin 5 promoter to target epithelial cells (61). Development of this animal model allowed investigators to better understand the role of oncogenes, c-myc, kras, and AKT, in epithelial ovarian cancer *in vivo*. However, because the keratin 5 promoter targets epithelial cells from multiple organs, ovaries were infected in culture and then transplanted back into recipient mice.

Ovarian transplant procedures are difficult and time consuming. Thus this may not be an efficient experimental model to use, although these initial studies developed some of our earlier understanding of epithelial ovarian cancer *in vivo*.

Another model used to understand epithelial ovarian cancer was developed by Connolly et al. who targeted simian virus 40 T antigen (SV40 TAg) to the epithelial ovarian surface by using the mullerian inhibitory substance type II receptor (MISIIR) promoter (62). Approximately 50% of female mice developed ovarian tumors that mimicked histology of the serous type. However, these mice die between 6-13 weeks of age and are infertile, making it almost impossible to establish a permanent line of transgenic animals. Furthermore, these animals develop extremely aggressive tumors early in life which does not mimic what is seen in women.

To develop an animal model that more accurately represents human ovarian cancer, Flesken-Nikitin et al. developed a novel experimental technique to understand the function of tumor suppressor genes, in this case p53 and Rb (63). Mice which have loxp sites inserted into a gene of interest were used in these experiments. Deletion of the specific gene within the ovarian epithelial cells was obtained by injecting adenoviruses, which express cre recombinase (Cre) driven by the cytomegalovirus (CMV) promoter, under the bursa of the ovary. This experimental technique has been picked up by several laboratories to generate mouse models.

Since the work of Connolly et al. and Flesken-Nikitin et al., several laboratories have generated other animal models by utilizing the conditional knockout system. Wu et al. used this technique to knock-down PTEN and adenomatous polyposis coli (APC) expressed in the OSE. These transgenic mice form tumors that specifically mimic ovarian endometrioid adenocarcinomas (64). Perhaps the most recent generation of a mouse model is by Joanne Richard's laboratory (65). This model uses the MISIIR promoter to drive excision of the PTEN and KRAS genes. This animal model forms serous ovarian carcinomas.

The past decade has brought new insight to the understanding of ovarian epithelial tumorigenesis *in vivo*. However many of these animal models develop very aggressive tumors early in life. This does not reflect the physiology of ovarian tumor progression in women because ovarian cancer is far more frequently diagnosed in older post-menopausal women. Furthermore

administration of cre recombinase by intra-bursal adenovirus administration may result in observations that deviate from the pathology seen in humans (66). Caution should be taken when developing or using these developed animal models to study ovarian epithelial tumorigenesis.

3.4 Estrogen and Epithelial Ovarian Cancer

The role of estrogen in breast tumorigenesis has long been established. Today women with breast cancer are often treated with aromatase inhibitors or estrogen receptor antagonists to effectively treat breast cancer. However, the role of estrogen in ovarian cancer remains less clear.

Several epidemiological studies have shown that post-menopausal women receiving hormone replacement therapies have an increased risk of ovarian cancer (67-70). Both estrogen or estrogen plus progesterone hormone replacement therapies are known to increase a woman's risk. However women of reproductive age taking hormones as oral contraceptives have a decreased risk of ovarian cancer. This may seem paradoxical at first but post-menopausal women have very high levels of other circulating hormones such as FSH and LH. These epidemiological data may indicate that ovarian tumorigenesis is driven by estrogen in combination with high levels of FSH and/or LH.

Three phase II studies have been carried out testing the effectiveness of letrozole, an aromatase inhibitor, as a treatment for ovarian cancer. Collectively, these studies indicate that a population of women would benefit from letrozole treatment. Phase II trial for ovarian cancer treatment studies enroll women who have stage III or IV cancer because the lack of diagnostic techniques limits enrolling many women with stage I or II cancer. Letrozole may inhibit ovarian tumorigenesis in early stages but studies have not clearly addressed this possibility.

3.5 Specific Aims

Recently a transgenic mouse model was developed in the lab. This mouse model lacks expression of ERα within the pituitary resulting in a lack of negative estrogen feedback on the hypothalamus-pituitary-ovarian axis. Furthermore, this conditional knockout mouse model, termed $ER\alpha^{d/d}$, maintains ERα within the ovary and over-expresses aromatase in the ovary. These phenotypes result in high circulating levels of E, P, luteinizing hormone (LH) and testosterone (T). Based on these observations we formulated the following aim:

Aim 3. To investigate the role of estrogen acting via ERα in the formation of ovarian epithelial tumors.

Determine the efficiency of letrozole, an aromatase inhibitor, in blocking ovarian tumorigenesis.

CHAPTER 4: The Role of Estrogen Receptor Alpha in the Ovary During Ovarian Tumorigenesis

4.1- Abstract

The etiology of ovarian cancer is poorly understood, mainly due to the lack of an appropriate experimental model for studying the onset and progression of this disease. Recently we have developed a mouse model in which aberrant estrogen receptor alpha (ERα) signaling in the hypothalamo-pituitary-ovarian axis leads to ovarian tumorigenesis. In this mouse model, termed $ER\alpha^{d/d}$, a conditional deletion of ERα gene occurred in the anterior pituitary, but ERα expression remained intact in the hypothalamus and the ovary. Selective ablation of ERα in the pituitary created a systemic hormonal imbalance in these mice. Loss of the negative-feedback regulation of estrogen (E) in the pituitary led to elevated levels of LH. LH hyperstimulation of ovarian cells resulted in elevated steroidogenic activities, leading to high circulating levels of E (64 pg/ml), P (9 ng/ml), LH (2.3ng/ml), and T (395 ng/ml). The $ER\alpha^{d/d}$ mice exhibited formation of ovarian tumors with 100% penetrance, starting at 5 months of age. These tumors grew to about 11 mm in size by 8 months of age and most mice died by 11-12 months. Immunohistochemical analyses revealed that the cells within the ovarian tumors of $ER\alpha^{d/d}$ mice are characterized by intense expression of cytokeratin 8 and 19 as well as nuclear staining of Wilms tumor 1, a well known marker of ovarian tumorigenesis, and paired homeobox 8 (PAX8), a marker of serous ovarian tumors. We investigated the ovarian cellular localization of E production. Aromatase expression was 16-fold higher in ovarian tumors compared to normal ovaries and was found to be expressed by the ovarian interstitial cells. Since E is known to drive ovarian tumorigenesis in other organs such as the breast, we treated mice with letrozole, an aromatase inhibitor. Letrozole treatment significantly decreased ovarian tumor burden when compared to sham treated controls. We have developed an animal model, which will serve as a powerful tool for exploring the involvement of steroid hormone signaling pathways in the etiology of ovarian tumors.

4.2- Introduction

Ovarian cancer remains the most deadly gynecological cancer among women, largely because the majority of women are diagnosed after the cancer has become metastatic (47). A lack in early diagnosis indicates a deficiency of adequate tools to detect this disease in its infancy. The development of animal models with ovarian cancer will give medical investigators insight into the establishment and progression of this disease *in vivo*. This information is critical for developing diagnostic tools to detect ovarian cancer in its early stages, an essential step in lowering the death rate due to ovarian cancer.

Several types of ovarian tumors exist depending on the cellular origin of the tumor. Epithelial ovarian tumors account for approximately 90% of all ovarian cancers in the western world (59). The majority of these cancers fall into 1 of 4 main histotypes: serous, endometrioid, mucinous, or clear cell. In the western world serous ovarian tumors account for 70% of all ovarian cancers (60).

Because of progressive research in the past decade, biochemical markers have been identified to determine the histotype of epithelial ovarian cancer. The up-regulation of Wilms tumor 1 (WT1) along with ovarian tumor expression of paired homeobox 8 (PAX8) are used to diagnose serous ovarian tumors from other epithelial ovarian tumor types (71).

The incidence of ovarian serous carcinoma increases as a woman ages, with an average patient age of 56 years (72). Several epidemiologic studies suggest that there is an increased risk of ovarian cancer for post-menopausal women taking hormone replacement therapy, either estrogen or estrogen in combination with progesterone (67-70). This leaves the possibility that high steroid hormone levels, in combination with the high gonadotropin levels characteristic of post-menopausal women, may drive ovarian tumorigenesis.

The etiology of ovarian tumorigenesis remains largely unknown. Undoubtedly, this is a result in a lack of adequate animal models to use for *in vivo* investigation of this disease. To date, there have been a few mouse models created that develop epithelial ovarian tumors. The existing mouse models for ovarian epithelial tumors involve inactivation of various tumor suppressors

such as PTEN, APC, p53, and RB, or over-expression of known tumor promoters such as c-myc, Kras, and Akt, through intrabursal administration of adenoviral vectors (61-66). Although these tumor models have provided valuable information regarding the gene dysfunction required for tumorigenesis, the aggressiveness and rapidity of tumor formation in these models limit identification of early biomarkers. Furthermore, gene deletion or over-expression via bursal injections of adenoviruses does not allow establishment of a permanent line for therapeutic studies. Thus, despite generating ovarian tumors, these mouse models have limited usefulness because of their dependence on strong oncogenic signals that may not reflect the true progression of genetic changes that lead to ovarian cancer in humans.

In this study, we introduce a transgenic mouse model, $ER\alpha^{d/d}$ mice, in which ovarian tumors develop with 100% penetrance. The onset and progression of tumorigenesis in these mice is considerably slower compared to other models that have been reported previously. In this model, conditional deletion of ERα in the anterior pituitary, without affecting its expression in the ovary, leads to a systemic hormonal imbalance that drives ovarian tumorigenesis. The $ER\alpha^{d/d}$ ovarian tumors over-express WT1 and PAX8, two known biochemical markers of human serous epithelial ovarian tumors. This novel animal model will be useful to examine epithelial ovarian tumorigenesis *in vivo*.

4.3- Results

Mature Adult $ER\alpha^{d/d}$ Mice Develop Ovarian Tumors with 100% Penetrance

We have created a transgenic mouse model that forms ovarian tumors by five months of age with 100% penetrance (Fig. 4.1A). These conditional knockouts of the ERα gene were created by employing the Cre-LoxP strategy. Transgenic mice expressing Cre under the control of progesterone receptor (PR) promoter, termed PR-cre mice, were crossed with mice harboring the 'floxed' ERα gene ($ER\alpha^{f/f}$) to create the $ER\alpha^{d/d}$ mice in which the ERα gene is deleted in cells expressing PR (38-39). The PR-cre transgenic mouse has been previously used to effectively knockdown gene expression in the uterus (25). Interestingly $ER\alpha^{d/d}$ mice form large

ovarian tumor masses but the global ERα knockout mice and $ER\alpha^{f/f}$ control mice do not develop ovarian tumors (Fig. 4.1B). Tumor masses progressively enlarge in the $ER\alpha^{d/d}$ mice with age and grow as large as 11 mm in size and weigh an average of 300 mg by eight months of age. Due to the burden of this large tumor mass on the stomach and intestines, $ER\alpha^{d/d}$ mice die between 8-11 months of age because they fail to eat.

The histology of these ovarian tumors has been analyzed (Fig. 4.1C). The stromal compartment of $ER\alpha^{d/d}$ ovarian tumors appears disorganized as early as 3 months of age. At 6 months of age $ER\alpha^{d/d}$ ovarian surface epithelium begins to invaginate within the ovarian tumor. By 11 months of age, when full-blown tumors arise, many cells with epithelial morphology can be seen within the $ER\alpha^{d/d}$ ovarian tumor.

Ovarian Tumor Cells in $ER\alpha^{d/d}$ Mice are Hyperproliferative and Express Elevated Levels of Phosphorylated AKT at Ser 473

To assess the proliferation status of cells within the ovarian tumors of $ER\alpha^{d/d}$ mice, we performed immunohistochemical analysis using an anti- proliferating cell nuclear antigen (PCNA) antibody, a well known proliferation marker. As expected $ER\alpha^{f/f}$ mouse ovaries revealed a high level of proliferation in the granulosa cells, but ovarian surface epithelial (OSE) cells remained quiescent. $ER\alpha^{d/d}$ mice at 6 months of age also possessed follicles with proliferating granulosa cells. However, in sharp contrast to $ER\alpha^{f/f}$ mouse ovaries, $ER\alpha^{d/d}$ ovarian tumors contained hyperproliferative surface epithelial cells as well as tumor cells within the ovary (Fig. 4.2A).

The phosphorylated AKT (p-AKT), the active form of the protein, is a critical player in cancer cell proliferation in multiple cancerous tissues. Ovarian cancer cells express high levels of p-AKT to promote tumor cell proliferation (73-74). To investigate the role that p-AKT might play in $ER\alpha^{d/d}$ ovarian tumor cell proliferation, we employed immunohistochemical analysis using an antibody against p-AKT. $ER\alpha^{f/f}$ ovaries express very low levels of p-AKT in the OSE. $ER\alpha^{d/d}$ ovarian tumors, however, express prominent expression of p-AKT in the OSE as well as

in tumor cells, the same cell types that are hyperproliferative as marked by PCNA (Fig. 4.2B). High expression of p-AKT in proliferating tumor cells suggests that p-AKT plays a role in ERα$^{d/d}$ ovarian tumor cell proliferation.

ERα$^{d/d}$ Mouse Tumors are Epithelial Cell Ovarian Tumors

Ovarian tumors can arise from multiple cell types within the ovary. Two of the most common ovarian tumors are granulosa cell tumors and epithelial cell tumors. To determine the type of ovarian tumor in the ERα$^{d/d}$ mice, we first employed immunohistochemical analysis using an antibody against anti-mullerian hormone (AMH), a well known marker for granulosa cells and granulosa cell tumors (75). Both ERα$^{f/f}$ and ERα$^{d/d}$ ovaries expressed AMH exclusively in granulosa cells within follicles (Fig. 4.3A). ERα$^{d/d}$ ovaries did not express AMH in the tumor cells indicating that the ovarian tumors are not of granulosa cell origin.

We next performed immunohistochemical analysis using anti-cytokeratin 8 antibody to determine if these tumors are of an epithelial cell type. ERα$^{f/f}$ mice of 3, 6, and 11 months of age express cytokeratin 8 exclusively in the OSE (Fig. 4.3B). ERα$^{d/d}$ mouse ovaries at 3 months of age express cytokeratin 8 exclusively in the OSE. However, OSE cells of ERα$^{d/d}$ mice at 3 months of age aggregate in multiple layers, an abnormality for the OSE. At 6 months of age, ERα$^{d/d}$ mouse ovaries begin to show cytokeratin 8 within the ovary indicating that the ovarian tumor contains epithelial cells. By 11 months of age nearly the entire ERα$^{d/d}$ mouse ovarian tumor expresses cytokeratin 8, indicating that this ovarian tumor is of epithelial cell origin.

ERα$^{d/d}$ Mouse Epithelial Ovarian Tumors Mimic Human Serous Epithelial Ovarian Tumors

It is well known that the majority of ovarian epithelial tumors are one of four different histotypes: serous, endometroid, mucinous and clear cell. The American Cancer Society reports that over 80% of all human epithelial ovarian tumors are characterized as serous and display characteristics of Fallopian tube epithelium. Recent studies have revealed biochemical markers

that will identify each histotype of ovarian epithelial cancer. Nonaka, et al. report that the expressions of WT1 together with PAX8, a protein normally expressed by the Fallopian tube epithelium, are characteristic specifically of the serous epithelial ovarian tumor (71). We employed these biochemical markers to further characterize the epithelial ovarian tumors of $ER\alpha^{d/d}$ mice.

WT1 is not expressed in the nucleus of any ovarian cell type of $ER\alpha^{f/f}$ mice. Abundant nuclear WT1 is expressed in both OSE and ovarian tumor cells of $ER\alpha^{d/d}$ mice (Fig. 4.4A). In addition, PAX8 is highly expressed in ovarian tumor cells but is absent in the OSE of $ER\alpha^{d/d}$ mice, an expression pattern that is similar to human serous ovarian cancer (Fig. 4.4B). In contrast, $ER\alpha^{f/f}$ mouse ovaries do not express PAX8, but the Fallopian tube epithelium exhibits high expression of nuclear PAX8. These data indicate that $ER\alpha^{d/d}$ ovarian tumors mimic human serous type of epithelial ovarian tumors.

$ER\alpha^{d/d}$ Mouse Epithelial Ovarian Tumors Express Similar Genes as Human Serous Epithelial Ovarian Tumors

We assessed possible similarities between $ER\alpha^{d/d}$ ovarian tumors and human serous tumors by analyzing mRNA expression of several genes known to be up-regulated in human serous tumors. Platelet derived growth factor receptor (PDGFR) is over-expressed in human serous ovarian tumors and is targeted in clinical trials as a treatment for ovarian cancers (85-86). $ER\alpha^{d/d}$ ovarian tumors, like human serous ovarian tumors, express high levels of PDGFR at 3, 6, and 11 months of age (Fig. 4.5A). Vascular cell adhesion molecule (VCAM) is found in the blood of cancer patients and has recently been proposed as a marker to detect early stages of ovarian cancer (87-88). Elevated VCAM mRNA levels are overexpressed in $ER\alpha^{d/d}$ ovarian tumors (Fig. 4.5B).

Human ovarian tumors that are sensitive to cisplatin have an increased expression of trophinin, a membrane protein that is associated with cell adhesion. Human ovarian tumors resistant to paclitaxel express elevated levels of clusterin, a protein of unknown function (89-90).

Interestingly ERα$^{d/d}$ ovarian tumors express elevated levels of both trophinin and clusterin (Fig. 4.5C&D). The precise molecular mechanism by which cisplatin and paclitaxel affect ovarian tumor growth is largely unknown. Employing ERα$^{d/d}$ mice to study ovarian tumorigenesis may aid in the discovery of underlying molecular mechanisms of these chemotherapies.

We next analyzed the gene expression profile in ERα$^{d/d}$ ovarian tumors and compared the molecular fingerprint of ERα$^{d/d}$ ovarian tumors to that of human serous epithelial ovarian cancer. RNA was isolated from ERα$^{d/d}$ ovarian tumors and subjected to microarray analysis. A list of 2620 genes were aberrantly expressed +/- 1.2 fold compared to ERα$^{f/f}$ ovaries. In order to compare this list to the molecular fingerprint of human serous epithelial ovarian cancer, three microarray studies comparing human serous ovarian adenocarcinoma to normal ovaries were exported from the Oncomine public database. These gene lists were compared to the list of aberrantly expressed genes in ERα$^{d/d}$ ovarian tumors using Ingenuity Pathway Analysis. This analysis indicated 25-40% similarity of aberrantly expressed genes in the ERα$^{d/d}$ ovarian tumors with that of human serous ovarian adenocarcinoma (Table 4.1). This is a remarkable similarity considering these mRNA fingerprints of ovarian cancer are in two different species. Clearly ERα$^{d/d}$ is a promising mouse model to study the human serous ovarian tumorigenesis.

Differential Loss of ERα in the HPO Axis of ERα$^{d/d}$ Mice Results in a Loss of Estrogen Negative Feedback on the HPO Axis

The finding that ERα$^{d/d}$ mice develop ovarian tumors while global ERα knockout do not develop these tumors remained a puzzling phenomenon until we investigated the expression of ERα in the HPO axis of ERα$^{d/d}$ mice. It is known that PR is expressed in all organs among the hypothalamus-pituitary-ovarian (HPO) axis (76). Along with PR ERα is also known to be expressed in these same organs (77). We determined if PR-cre was able to create a functional loss of ERα in the hypothalamus, pituitary and ovary of ERα$^{f/f}$ and ERα$^{d/d}$ mice.

In the hypothalamus of ERα$^{f/f}$ mice, ERα is expressed in the hypothalamus near the third ventricle, and this expression remains intact in the hypothalamus of ERα$^{d/d}$ mice (Fig. 4.6A&B).

Pituitary expression of ERα is wide-spread in the anterior lobe of the ERα$^{f/f}$ mice (Fig. 4.6C). Expression of ERα is nearly ablated in the ERα$^{d/d}$ mice leaving the possibility that these mice may experience a loss of estrogen negative feedback on the HPO axis (Fig. 4.6C). Interestingly, ERα within the ERα$^{d/d}$ mouse ovary remains intact (Fig. 4.6G&H). Specifically, the OSE and ovarian tumor cells maintain ERα expression. Clearly the pituitary is the only organ within the HPO axis to experience a loss of ERα in the ERα$^{d/d}$ mice.

ERα$^{d/d}$ Mice Have Elevated Levels of Circulating Hormones

We next evaluated serum steroid hormones and gonadotrophin levels in ERα$^{f/f}$, ERα$^{d/d}$, and ERα KO mice. Unexpectedly serum estradiol levels were notably increased in the ERα$^{d/d}$ mice compared to both ERα$^{f/f}$ and ERα KO mice (Table 4.2). Similarly, progesterone levels and testosterone levels also were markedly higher in the ERα$^{d/d}$ mice compared to both ERα$^{f/f}$ and ERα KO mice. LH levels in the ERα$^{d/d}$ mice remained higher than the ERα$^{f/f}$ mice but were comparable to ERα KO mice. Interestingly, serum follicle stimulating hormone (FSH) was not statistically different between ERα$^{d/d}$, ERα$^{f/f}$, or ERα KO mice. It is likely that the high hormone signature of the ERα$^{d/d}$ mice plays a role in driving ovarian tumorigenesis.

ERα$^{d/d}$ Mice Display Aberrant ERα Signaling within Ovarian Tumors

Because ERα$^{d/d}$ mouse ovaries maintain functional ERα and extreme high levels of serum estrogen, we evaluated ERα signaling in the ERα$^{d/d}$ mouse ovarian tumors. Phosphorylated ERα(p- ERα) is the active form of ERα and positively influences the transcriptional ability of both liganded and unliganded ERα (78-79). Phosphorylation of ERα, specifically at Ser-118, occurs in human breast tumors, is activated by the MAPK pathway and plays a functional role in human breast tumor tamoxifen resistance (80-81). We analyzed p-ERα expression at Ser-118 in ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors. Although p-ERα is

expressed at low levels in ERα$^{f/f}$ ovaries, ERα$^{d/d}$ ovarian tumors show extensive p-ERα expression in the OSE (Fig. 4.7C).

Aromatase is Highly Expressed in Interstitial Cells of ERα$^{d/d}$ OvarianTumors

ERα$^{d/d}$ mice have very high levels of circulating estradiol (Table 4.2). We investigated the cellular origin of estradiol in this mouse model. ERα$^{d/d}$ ovarian tumors express a 5-fold increase in aromatase mRNA when compared to ERα$^{f/f}$ ovaries (Fig. 4.8A). We next localized aromatase expression in interstitial cells within the ERα$^{d/d}$ ovarian tumors. Cells were released from ovarian tumors and then cultured *in vitro*. Immunocytochemistry was employed to co-localize aromatase with either cytokeratin, an epithelial cell marker, or vitmentin, an interstitial cell marker. Epithelial cells derived from the tumors stained positive for cytokeratin (Fig. 4.8B) but did not stain positive for aromatase (Fig. 4.8C). However, cells expressing vimentin (Fig. 4.8E) also stained positive for aromatase (Fig. 4.8F). These data confirm that aromatase is over-expressed in interstitial cells of ERα$^{d/d}$ ovarian tumors.

Letrozole Treatment Decreases Ovarian Tumor Volume

To address the role of aromatase in ERα$^{d/d}$ ovarian tumorgenesis, we treated mice with letrozole, an aromatase inhibitor, for three consecutive months. ERα$^{d/d}$ mice treated with letrozole developed tumors that were 60% smaller than ERα$^{d/d}$ mice treated with a sham control (Fig. 4.9 A&B). However, ovarian tumors in letrozole treated ERα$^{d/d}$ mice were still larger than the ERα$^{f/f}$ control ovaries. Letrozole effectively decreased estradiol serum levels without effecting LH serum levels in these mice (Fig. 4.9C). These data support the conclusion that letrozole effectively decreases ovarian tumor volume by specifically decreasing estradiol levels.

We analyzed the impact of letrozole on several biochemical markers of ovarian tumors. Letrozole significantly decreased expression of PDGFRα, VCAM, clusterin and trophinin (Fig.

4.10A-D). These molecules are known to be up regulated in human ovarian cancer and ERα$^{d/d}$ ovarian tumors. We also analyzed the expression of Wip1, a known target of estrogen in MCF7 cells. Letrozole also decreased the mRNA and protein expression of this estrogen regulated gene further confirming that letrozole effectively decreases tumor volume by regulating estrogen signaling in ERα$^{d/d}$ mice.

4.4- Discussion

It is predicted that 21,880 cases of ovarian cancer will be diagnosed in 2010 and 13,850 deaths will occur as a result of ovarian cancer (47). This high mortality rate is a direct result of late stage diagnosis for 60% of ovarian cancer cases. In sharp contrast, women diagnosed with stage I ovarian carcinoma have a 90% 5 year survival rate (91). Undoubtedly the mortality rate associated with ovarian cancer will improve with more early diagnoses of ovarian cancer. To improve the survival rate for women with ovarian cancer, we need tools that allow researchers to investigate the early stages of tumorigensis. In the present study we introduce a transgenic mouse model, ERα$^{d/d}$ mouse, that develops ovarian tumors at a slow rate due to a physiological perturbation and not a mechanical disturbance such as intrabursal injection of adenovirus. This mouse model presents an invaluable tool to study molecular markers and targets of early ovarian tumorigenesis.

Almost all ovarian carcinomas develop from epithelial ovarian tumors with serous tumors representing the most frequent histotype of epithelial tumors in humans. ERα$^{d/d}$ mouse ovarian tumors over-express WT1 much like human cases of serous tumors in which WT1 is used as a biochemical marker to diagnose serous epithelial ovarian tumors (92). However, WT1 is also up-regulated in other types of ovarian tumors such as sertoli cell ovarian tumors (93). To validate ERα$^{d/d}$ mouse ovarian tumors as a model for serous ovarian tumors we analyzed the expression of PAX8 within these ovarian tumors. PAX8 is present developmentally in the Mullerian duct and continues to be expressed in the epithelium of the fallopian tube after duct differentiation (94). Normally PAX8 is not expressed in the ovary but PAX8 is over-expressed in serous

epithelial ovarian carcinomas (95). ERα$^{d/d}$ mouse ovarian tumors also over-express PAX8. These data lead us to believe that ERα$^{d/d}$ mice will be a useful model to study serous ovarian tumorigenesis.

Although the fallopian tube epithelium is quiescent in ERα$^{d/d}$ mice, the hyper-proliferation status of the OSE is remarkable considering the OSE does not proliferate most of the time. Consistent with this observation we noted an over-expression of p-ERα (Ser 118) in the OSE of ERα$^{d/d}$ mice. This finding leads us to believe that ERα signaling is at the heart of epithelial ovarian tumorigenesis. Several epidemiologic studies indicate that women taking hormone replacement therapy during or after menopause, a time when women have high levels of gonadotropin levels, have an increased risk of ovarian cancer. Similar to postmenopausal women using hormone replacement therapy, ERα$^{d/d}$ mice experience high levels of steroid hormones along with high levels of gonadotropins. The unique eleveated hormone milieu of ERα$^{d/d}$ mice provides a unique model to better understand the role of these hormones in epithelial ovarian tumorigenesis.

Gene expression analysis indicates that many genes which are over expressed in human ovarian cancers are also up-regulated in ERα$^{d/d}$ ovarian tumors. These over expressed genes include PDGFRα, VCAM, clusterin and trophin. We next compared molecular finger prints of human serous ovarian cancer and ERα$^{d/d}$ ovarian tumors by using microarray analysis. Even though this comparison is between cancers from two different species there is still 40% homology in aberrantly regulated genes. These results support the conclusion that ERα$^{d/d}$ ovarian tumors mimic human serous ovarian cancer and these mice are an excellent tool for studying this pathophysiology.

ERα$^{d/d}$ mice have high circulating estradiol levels. We confirm that ERα$^{d/d}$ ovarian tumors over express aromatase and localize this aromatase expression to insterstitial cells within the ovarian tumor. The production of aromatase from the interstitial cells acts on epithelial cells to drive tumorigenesis. Estradiol is known to drive cancer development in other systems such as breast cancer. Using the ERα$^{d/d}$ animal model we show that letrozole treatment decreases ovarian

tumor volume by 60%. However tumor volume still remained larger than ERα$^{f/f}$ ovaries. In this study three month old mice were treated for three months. Even though three month old ERα$^{d/d}$ mice do not exhibit full-blown ovarian tumors, it is possible that the initial phases of tumorigenesis have already begun. Future studies will address the ability of letrozole to completely inhibit ovarian tumor formation. Our initial experiments clearly indicate that letrozole effectively decreases tumor volume. Clearly more clinical studies are needed to determine if there is a population of women with ovarian cancer that can benefit from this treatment.

In summary, we have generated a novel transgenic mouse model, ERα$^{d/d}$ mouse, which develops a slow progression of epithelium ovarian tumorigenesis. Due to a lack of estrogen negative feedback on the HPO axis at the level of the pituitary, these mice secrete high levels of steroid hormones and LH, a hormone milieu that mimics a post-menopausal women taking hormone replacement therapy. Using established biochemical markers, we have determined that these ovarian tumors over-express similar molecules to that of women with serous ovarian cancer. Furthermore treatment of these mice with letrozole significantly decreases ovarian tumor volume establishing ERα$^{d/d}$ mice as a hormone dependant murine model of ovarian tumorigenesis. Undoubtedly this unique animal model will be a powerful tool for studying the early events of ovarian tumorigenesis.

4.5- Materials and Methods

Hormone Assay

All hormone assays were completed by the University of Virginia Center for Research in Reproduction Ligand Assay and Analysis Core. Statistical significance was determined on SAS program using the Tukey procedure to control for comparison-wise error rate. Significance cutoff value of $p<0.05$ was determined to be statistically significant.

Immunohistochemistry

Immunohistochemistry was carried out as previously described. Specfic antibodies wer obtained from the following: cytokeratin 8 (Developmental Studies Hybridoma Bank -TROMA I), cytokeratin 19 (Developmental Studies Hybridoma Bank -TROMA III), PCNA (Santa Cruz sc-56), AMH (Compliments of Wylie Vale), WT1 (Santa Cruz sc-846), PAX8 (Proteintech group 10336-1-AP), calretinin (Invitrogen 180291), Ber-EP4 (DAKO), aromatase (Abcam ab35604) vimentin (Sigma Aldrich V5255), PPM1D/Wip1 (Sigma Aldrich HPA022277).

Immunocytochemistry

Ovarian tumor cells were cultured in 2 or 4 well chamber slides. Cells were fixed with 10% formalin solution for 10 minutes. Cells were washed with HBSS then treated with 25% triton X-100 (Sigma Aldrich) in PBS for an additional 10 minutes. Cells were washed with HBSS and exposed to a blocking serum for 1 hour. Cells were then treated with primary antibodies and incubated at 4^0C overnight. Cells were then washed with PBS and exposed to cy3 or cy5 conjugated secondary antibodies for 1 hour at room temperature.

Isolation of Ovarian Cells

Ovarian tumors were removed from mice after euthanasia with carbon dioxide. Ovarian tumors were either digested with 6 g/liter dispase (Invitrogen) and 25g/liter pancreatin, or 0.5 g/liter collagenase in Hanks balanced salt solution (HBSS). After incubation for 1 hour at 37 °C, the tubes were vortexed for 10-12 s until the supernatant became turbid with dispersed cells. The contents of the tube were then passed through a 80-μm gauze filter (Millipore). Cells were resuspended in Dulbeccos modified Eagle's Medium-F12 medium (DMEM-F12; with 100 unit/liter penicillin, 0.1 g/liter streptomycin, 1.25 mg/liter fungizone) containing 10% heat-inactivated fetal calf serum and live cells were assessed by trypan blue staining using a hemocytometer. Cells were seeded in 2 or 4 well chamber slides. The unattached cells were removed by washing with HBSS, and cell culture was continued for 48 hrs after addition of fresh medium.

Silastic capsule implant

Silastic capsules were made by sealing silastic laboratory tubing (1.57 mm I.D. x 2.41 mm O.D.) with medical adhesive silicone type A (Dow Corning). Femara 2.5 mg tablets (Novartis) containing letrozole were ground with a mortar and pestle. Capsules were filled with .8 mg of Femara tablets and sealed with medical adhesive silicone type A.

Mice treated with analgesic (buprenorphine 0.05 mg/kg s.c.) 1 hour prior to surgery. Mice were anesthetized with ketamine/xylazine 87 mg/kg; 15 mg/kg. A small incision through the skin was made dorsally just below the neck and the silastic capsule was inserted underneath the skin. The incision was held together with wound clips until healed. After 3 months of exposure to either empty silastic capsules (sham control) or silastic capsules containing letrozole mice were euthanized with carbon dioxide and ovarian tumors were fixed or frozen.

4.6- Figures and Tables

Fig. 4-1 A

Fig. 4-1 B

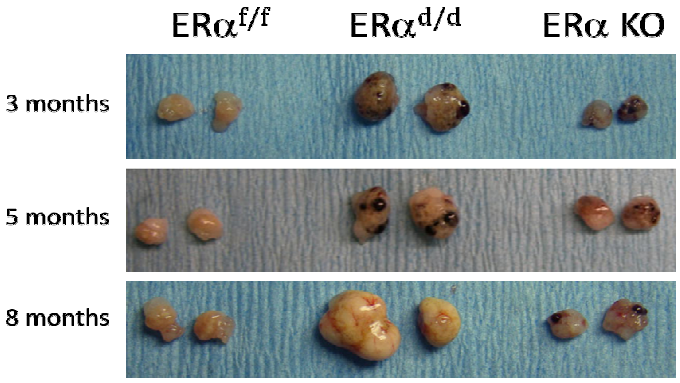


Fig. 4-1 C

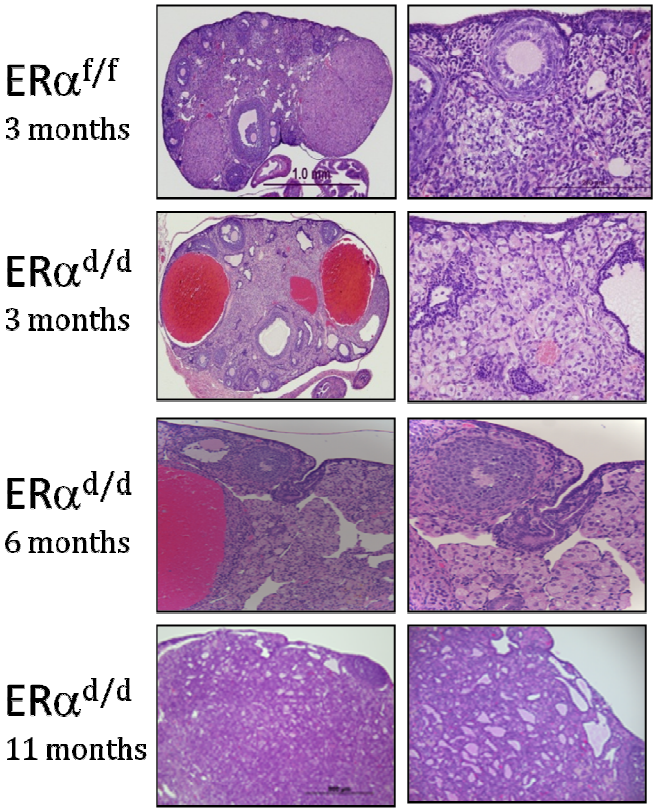


Fig. 4.1: ERα$^{d/d}$ Mice Display Ovarian Tumors by 5 Months of Age. (A) Bilateral ovarian tumors of ERα$^{d/d}$ mouse at 11 months of age. (B) Gross morphology of ERα$^{d/d}$, ERα$^{f/f}$, and ERα global knockout mouse (KO) ovaries at 3, 5, and 8 months of age. (C) Histological sections of ERα$^{f/f}$ ovaries at 3 months of age and ERα$^{d/d}$ ovarian tumors at 3, 6, and 11 months of age. Sections were stained with hemotoxylin and eosin and pictures were taken at 4x and 10x.

Fig. 4-2 A

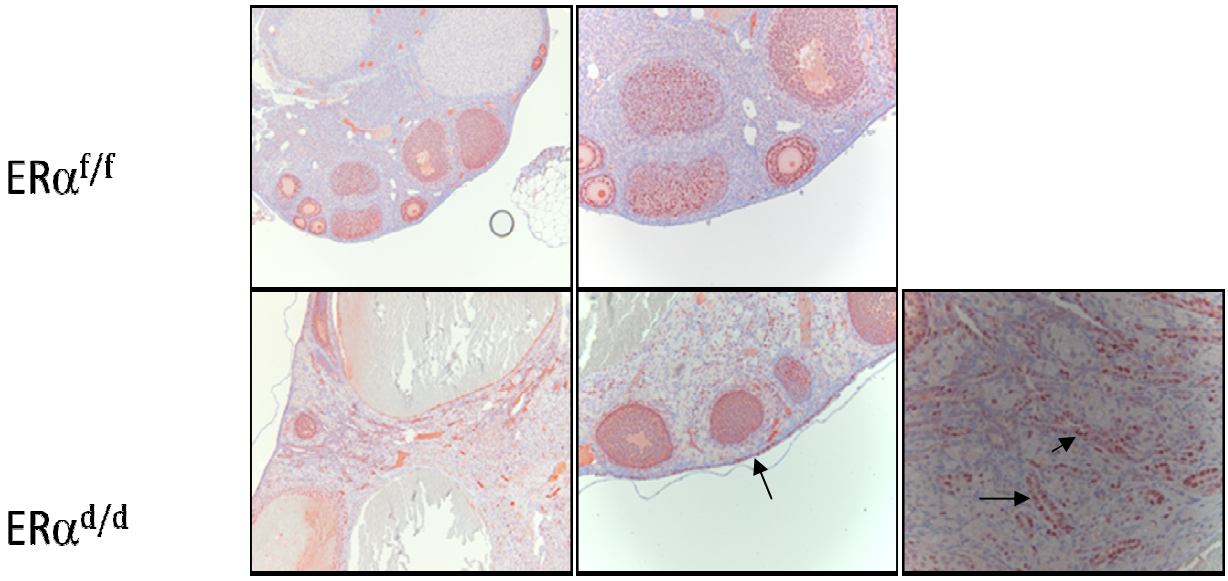


Fig. 4-2 B

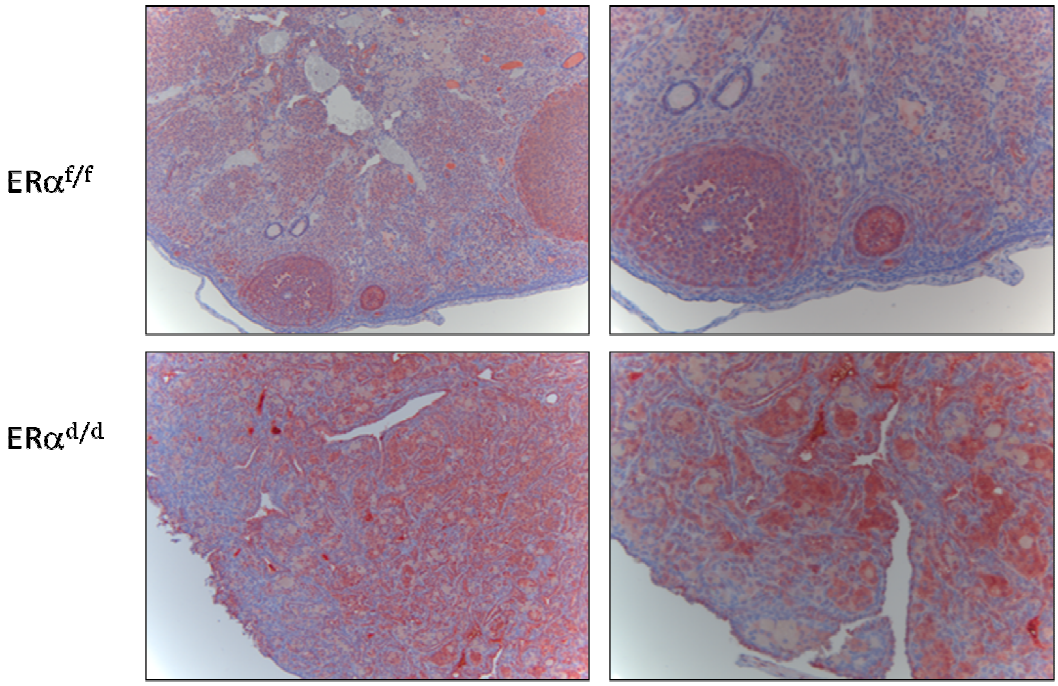


Fig. 4.2: ERα$^{d/d}$ Ovarian Tumors are Hyperproliferative. (A) Immunohistochemistry of ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors at 6 months of age using anti-PCNA antibody. Arrows point to hyperproliferative OSE and tumor cells in ERα$^{d/d}$ ovarian tumors. (B) Immunohistochemistry of ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors at 11 months of age using anti-p-AKT antibody (Ser 473).

Fig. 4-3 A

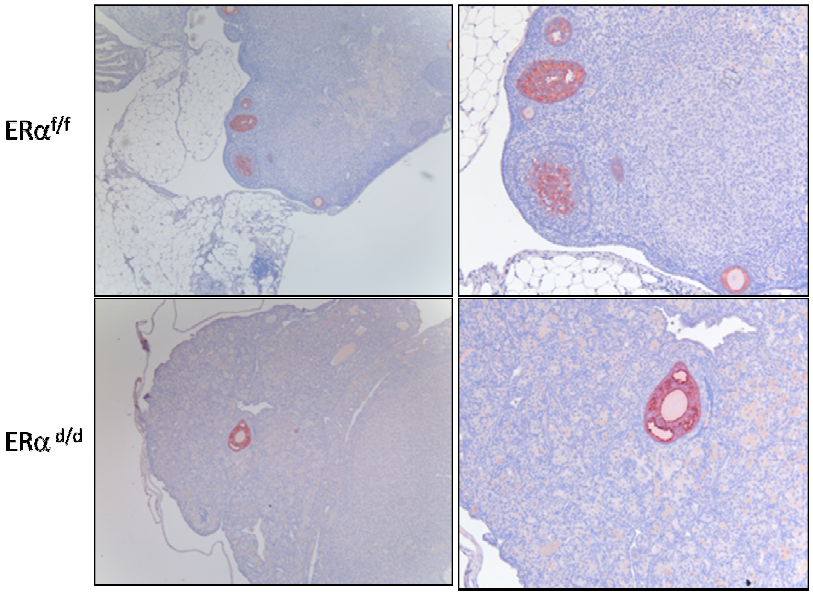


Fig. 4-3 B

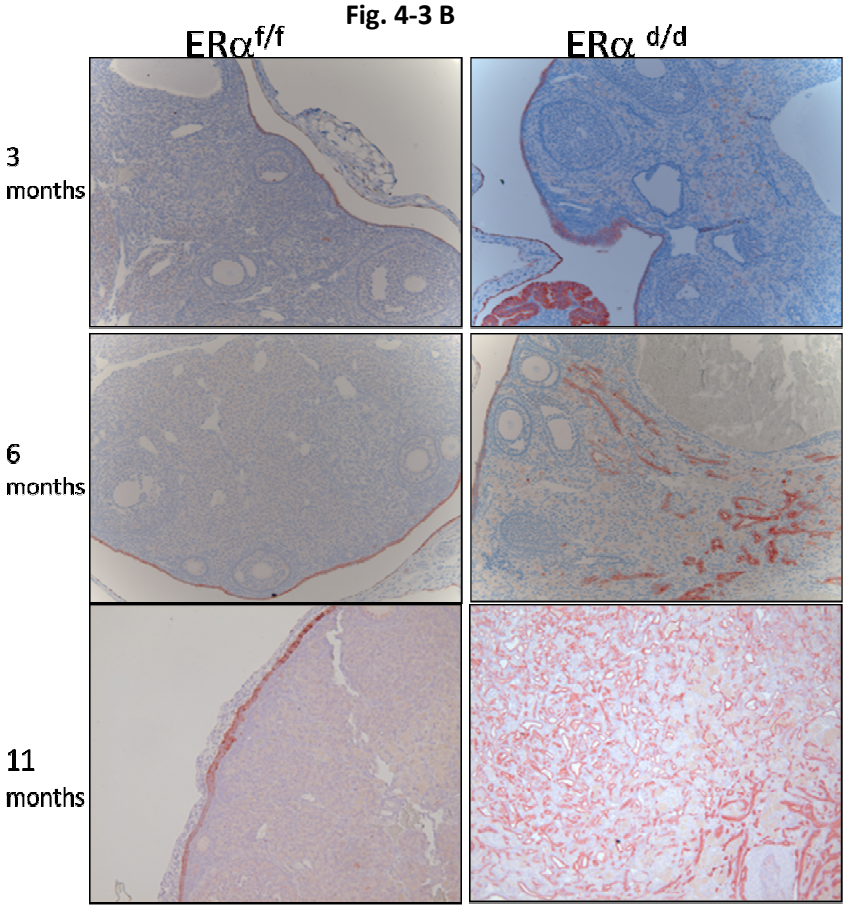
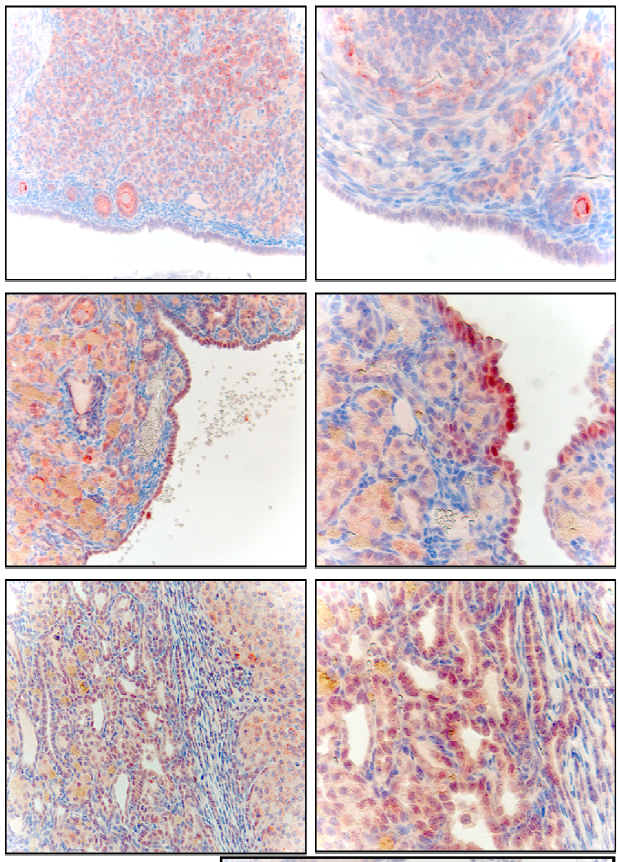


Fig. 4.3: ERα$^{d/d}$ Ovarian Tumors are Epithelial Cell Tumors Not Granulosa Cell Tumors.
(A) Immunohistochemistry of ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors at 11 months of age using an antibody against AMH.
(B) Immunohistochemistry of ERα$^{f/f}$ ovaries at 3 months of age and ERα$^{d/d}$ ovarian tumors at 3, 6, and 11 months of age using anti-cytokeratin 8 antibody.

Fig. 4-4: ERα$^{d/d}$ Ovarian Tumors Express Elevated Biochemical Markers, WT1 and PAX8, of Human Serous Epithelial Ovarian Tumors.
(A) Immunohistochemistry of ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors at 11 months of age using an antibody against WT1. Pictures are taken at 20x and 40x.

(B) Immunohistochemistry of ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors at 11 months of age using an antibody against PAX8. Pictures for ERα$^{f/f}$ ovaries are taken at 10x and 20x. Pictures for ERα$^{d/d}$ ovarian tumors are taken at 20x and 40x.

Fig. 4-4 A

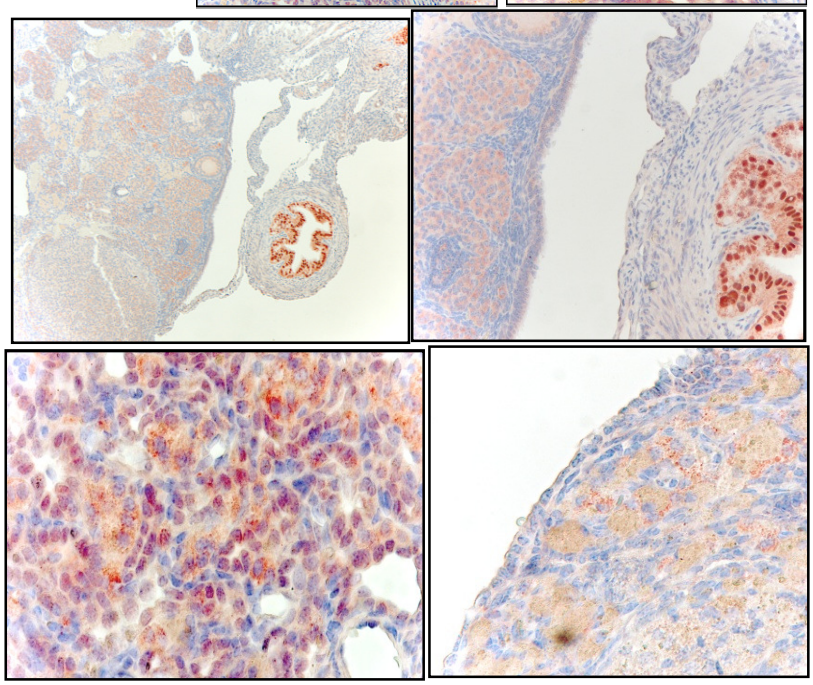


ERα$^{f/f}$

ERα$^{d/d}$

ERα$^{d/d}$

Fig. 4-4 B



ERα$^{f/f}$

ERα$^{d/d}$

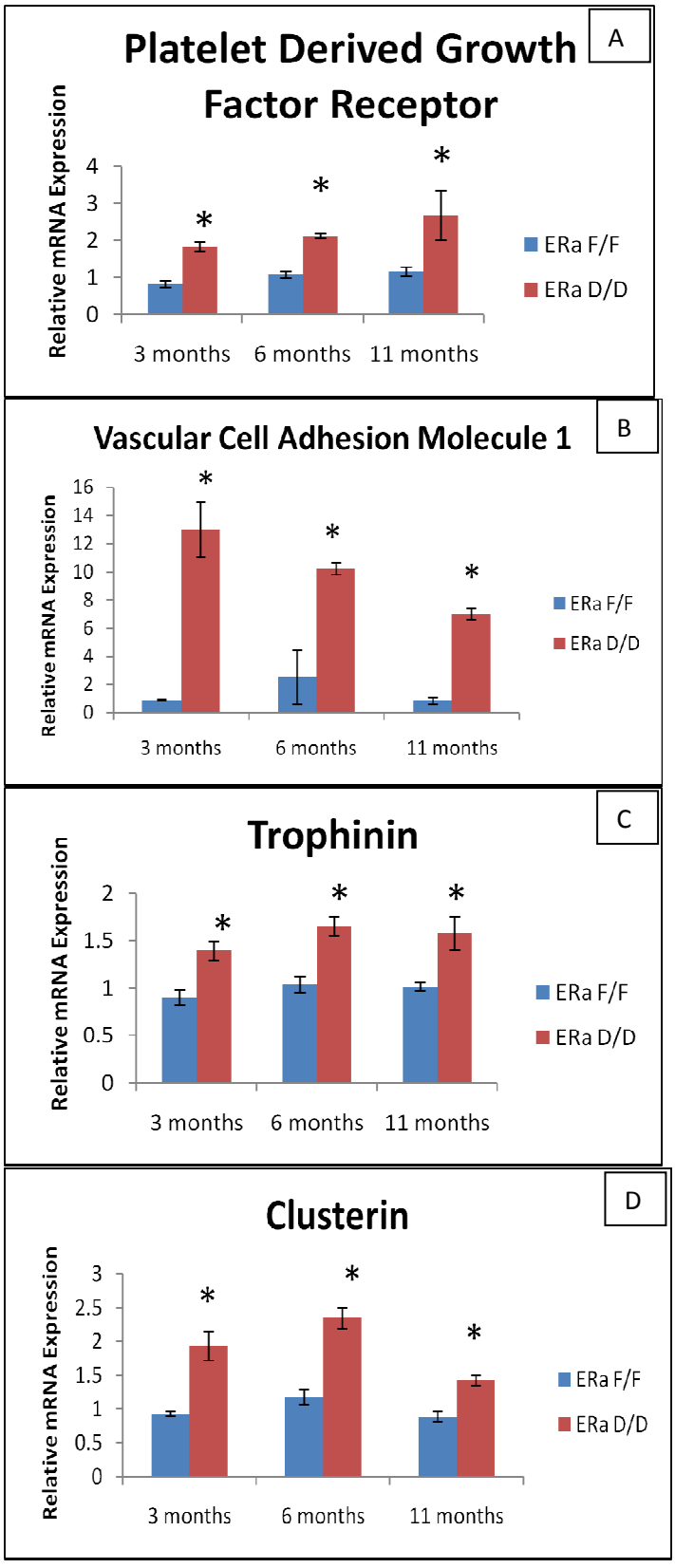


Fig. 4.5: ERα$^{d/d}$ Ovarian Tumors Express High Levels of Genes Known to be Up-regulated in Human Ovarian Tumors. ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors at 3, 6, and 11 months of age are isolated. Gene expression of PDGFRα (A), VCAM (B), trophinin (C), and clusterin (D) is measured by qPCR analysis of mRNA isolated from ovaries or ovarian tumors. * indicates a significant difference in gene expression between mice with different genetic backgrounds at a p-value cutoff of 0.05 using a student's t-test.

Study	Number of aberrantly regulated genes common between human and mouse	Percent of aberantly regulated genes in mouse that are common with human
Adib, TR. et al. (2004) Br J Cancer. 90(3): 686-92.	664	25%
Hendrix, ND. et al. (2006) Cancer Res. 66(3): 1354-62.	937	36%
Lu, KH. et al. (2004) Clin Cancer Res. 10(10): 2968-76.	1048	40%

Table 4.1: Microarray Analysis indicates a High Level of Similarity Between Aberrantly Expressed Genes in ERα$^{d/d}$ Ovarian Tumors and Human Ovarian Serous Adenocarcinoma. ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors are collected, mRNA extracted and subjected to microarray analysis. A list of 2620 aberrantly expressed genes +/- 1.2fold is generated. This list is compared to gene lists from 3 studies stored in the Oncomine database which compare gene expression in normal human ovaries vs. human ovarian serous adenocarcinoma. 25-40% of genes aberrantly expressed in ERα$^{d/d}$ ovarian tumors are common in human ovarian serous adenocarcimoma.

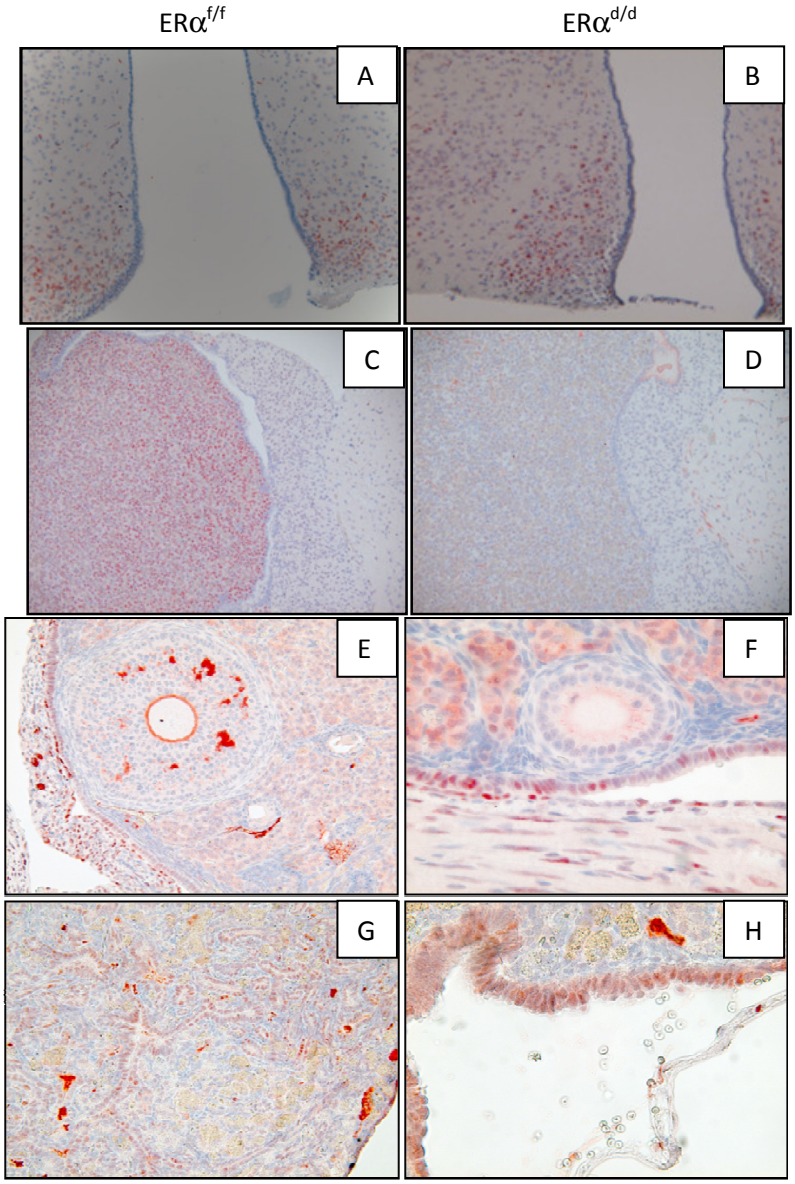


Fig. 4.6: ERα$^{d/d}$ Mice have Pituitary ERα Loss of Function but Maintain ERα Function in the Hypothalamus and Ovary. Histological sections of ERα$^{f/f}$ (A) and ERα$^{d/d}$ (B) hypothalami immunostained for ERα protein. Histological sections of ERα$^{f/f}$ (C) and ERα$^{d/d}$ (D) pituitaries immunostained for ERα protein. Histological sections of ERα$^{f/f}$ ovaries (E&F) and ERα$^{d/d}$ ovarian tumors (G&H) immunostained for ERα protein.

Hormone	ERa$^{f/f}$	ERa$^{d/d}$	ERa KO
Estradiol (pg/ml)	7.03 +/- 0.84 ^{A}	63.51 +/- 36.30 ^{B}	19.28 +/- 2.61 ^{C}
Progesterone (ng/ml)	1.94 +/- 0.61 ^{A}	8.78 +/- 0.77 ^{B}	1.58 +/- 0.44 ^{A}
LH (ng/ml)	.17 +/- 0.12 ^{A}	2.31 +/- 0.51 ^{B}	2.90 +/- 0.43 ^{B}
FSH (ng/ml)	6.30 +/- 1.58 ^{A}	2.92 +/- 0.57 ^{A}	4.66 +/- 0.27 ^{A}
Testosterone (ng/ml)	3.01 +/- 1.35 ^{A}	394.85 +/- 59.058 ^{B}	32.01 +/- 11.39 ^{C}

Table 4.2: Serum steroid hormone and gonadotrophin concentrations in ERα$^{d/d}$, ERα$^{f/f}$, and ERα KO mice. Hormone levels are displayed as the average +/- s.e.m. Tukey analysis was utilized for statistical signficance. Different letters indicate statistical difference at p value cutoff of 0.05.

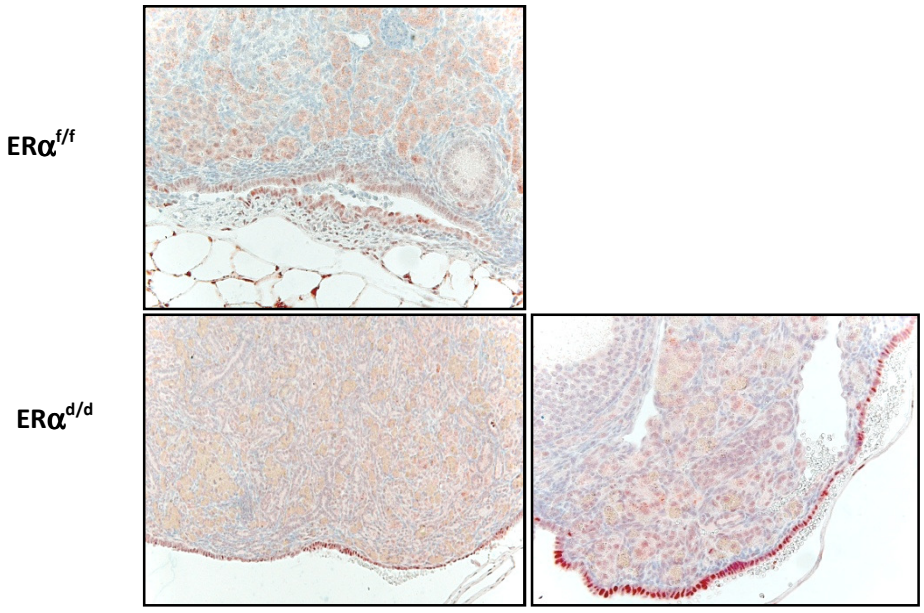


Fig. 4.7: ERα$^{d/d}$ ovarian tumors express high levels of p- ERα Immunohistochemistry of ERα$^{f/f}$ ovaries and ERα$^{d/d}$ ovarian tumors at 11 months of age using an antibody against p-ERα (Ser 118).

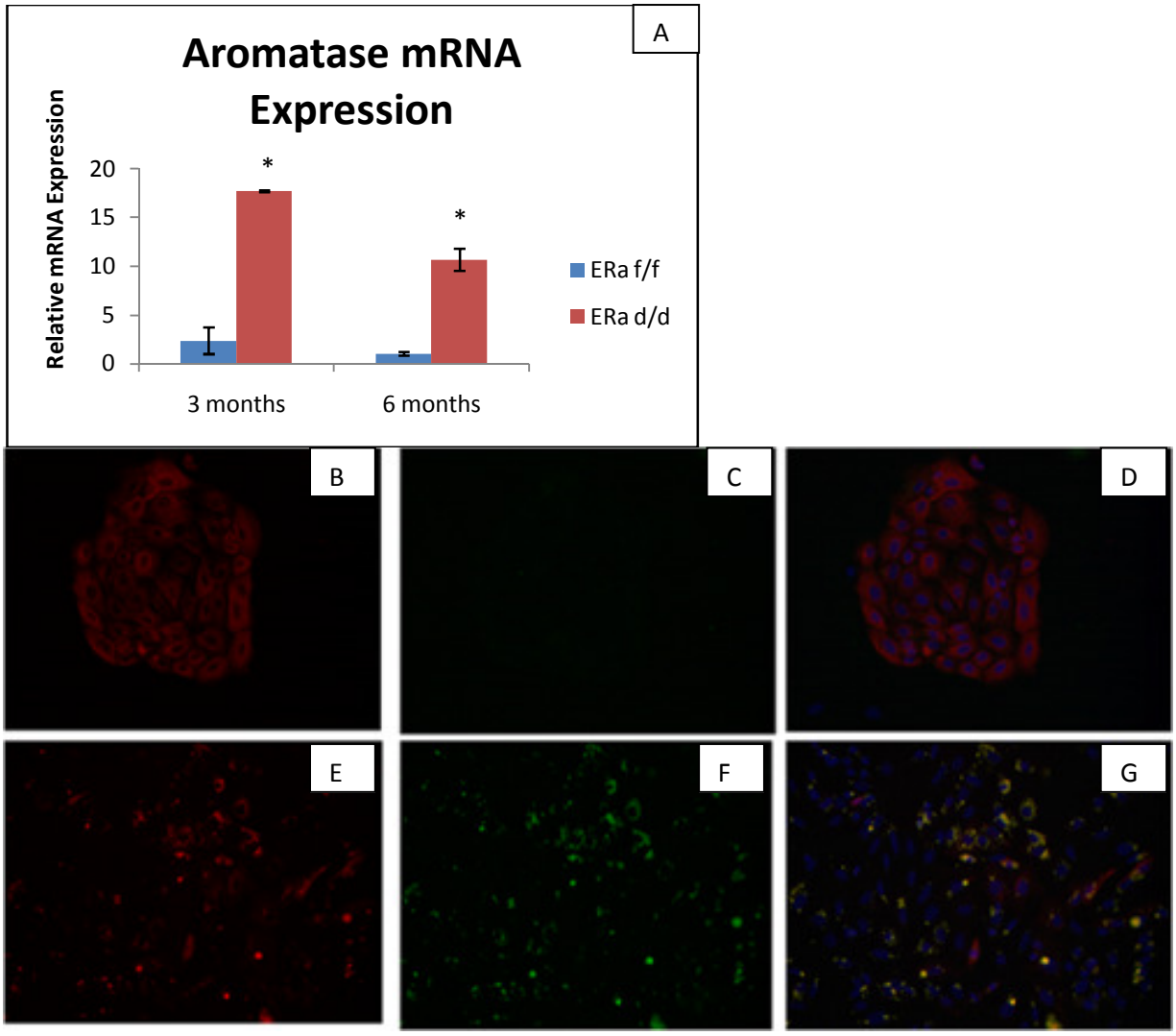


Fig. 4.8: ERα$^{d/d}$ Ovarian Tumors Interstitial Cells Express High Levels of Aromatase. (A) QPCR indicating higher mRNA levels of aromatase in ERα$^{d/d}$ compared to ERα$^{f/f}$ ovaries. ERα$^{d/d}$ ovarian tumors are digested and single cell suspensions are cultured for 48 hours. Epithelial cells are stained with anti-cytokeratin 8 (A) and anti-aromatase (B) antibodies. Overlap of A and B is represented in picture C. Dapi counterstain is blue. Interstitial cells are stained with anti-vimentin (E) and anti-aromatase (F) antibodies. Overlap of E and F is represented in picture G. Yellow indicates co-localization of vimentin and aromatase. * indicates a significant difference in gene expression between mice with different genetic backgrounds at a p-value cutoff of 0.05 using a student's t-test.

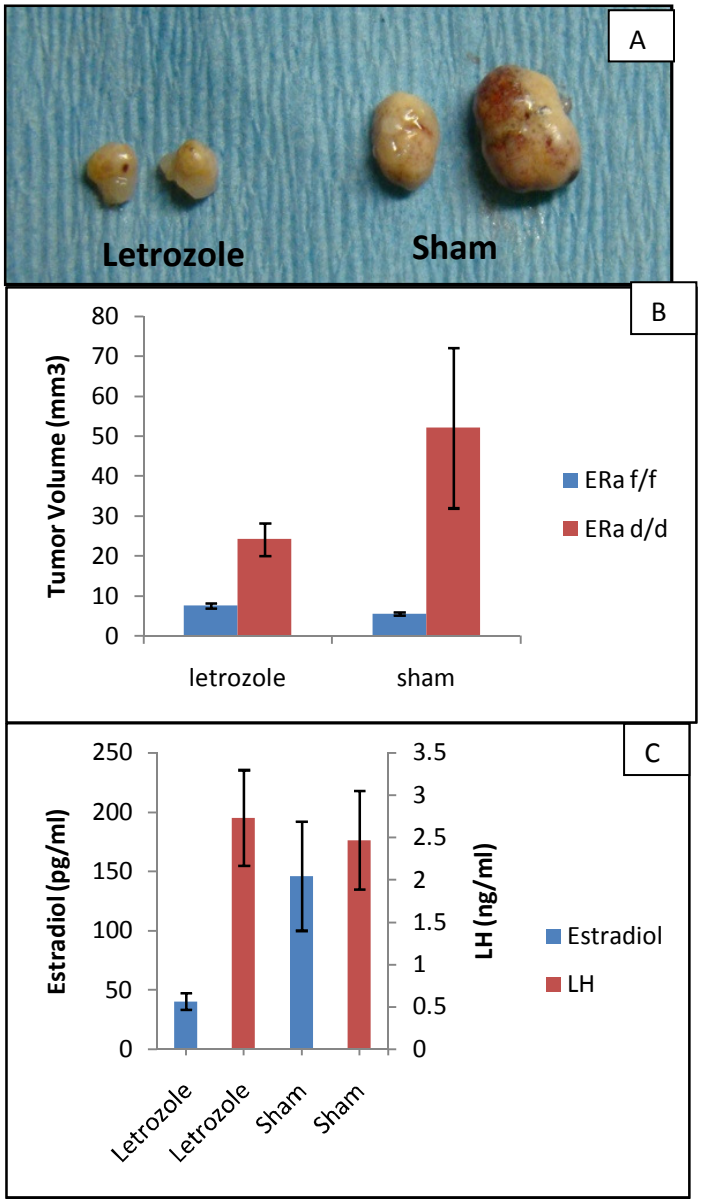


Fig. 4.9: Letrozole Treatment, an Aromatase Inhibitor, Decreases ERα$^{d/d}$ Ovarian Tumor Volume. Three month old ERα$^{d/d}$ and ERα$^{f/f}$ mice were treated with either letrozole filled silastic capsules or empty capsules (sham) for a period of three months. Gross morphology comparing ERα$^{d/d}$ ovarian tumors after treatment with letrozole (left) ERα$^{d/d}$ ovarian tumors untreated (right). Ovarian tumor volume of ERα$^{d/d}$ mice treated with letrozole is 60% smaller on average than ovarian tumor volume of ERα$^{d/d}$ mice untreated, p=0.02(B). Letrozole treatment is effective in reducing serum estradiol levels but does not decrease LH levels (C).

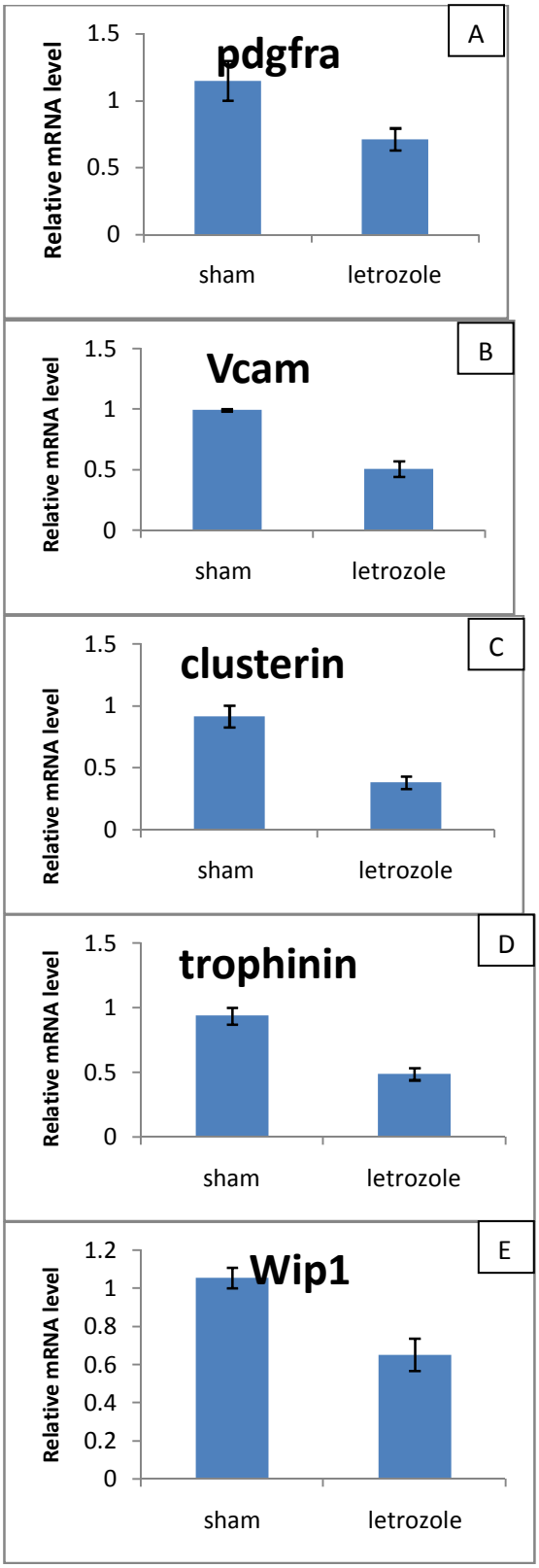


Fig. 4.10: Letrazole Treatment Decreases Expression of ERα$^{d/d}$ Ovarian Tumors Genes that are Known to be Up-regulated in Human Ovarian Tumors. ERα$^{d/d}$ mice are treated with letrozole or sham silastic capsules for three months. Gene expression of PDGFRα (A), VCAM (B), trophinin (C), clusterin (D) and Wip1 (E) is measured by qPCR analysis of mRNA isolated from ovarian tumors of untreated ERα$^{d/d}$ mice or ERα$^{d/d}$ mice treated with letrozole.

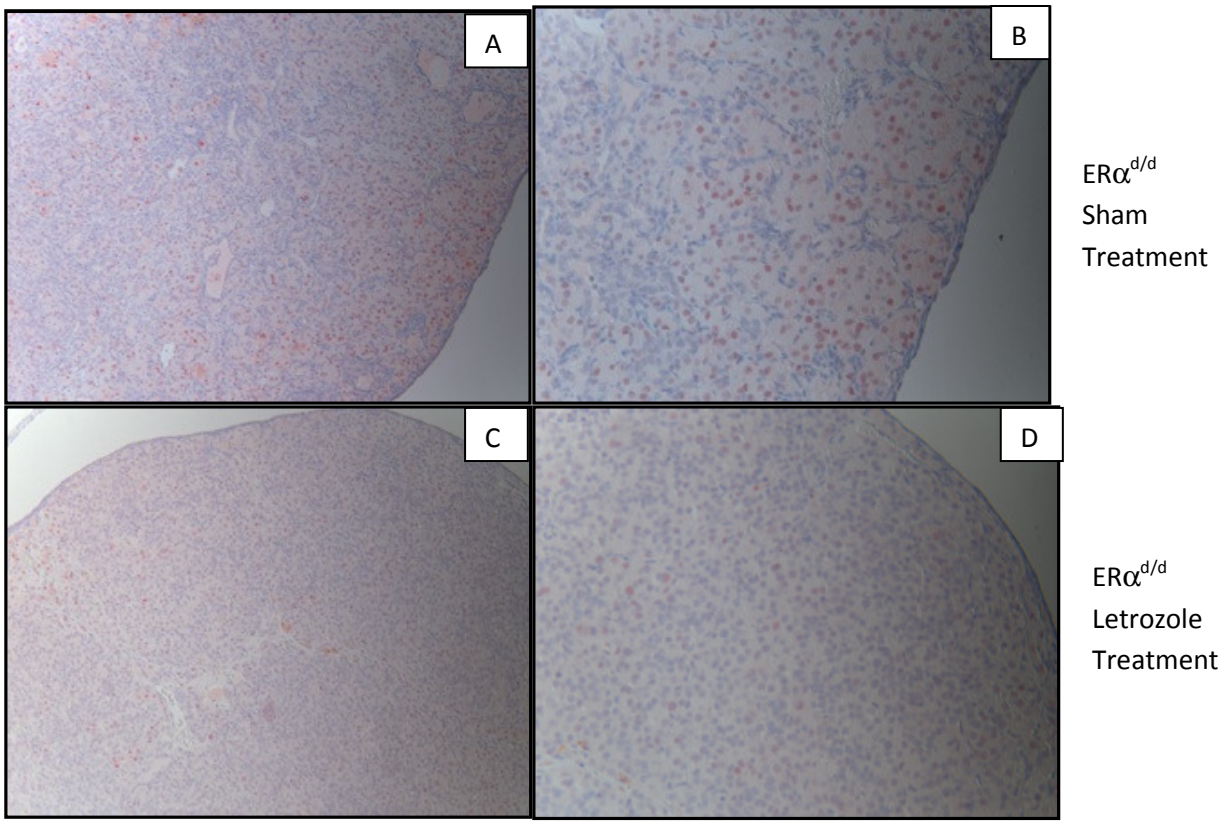


Fig. 4.11: ERα$^{d/d}$ Ovarian Tumors Treated with Letrozole Express Lower Levels of Wip1 protein, an Estrogen Regulated Gene. ERα$^{d/d}$ mice are treated with letrozole or sham silastic capsules for three months. ERα$^{d/d}$ untreated ovarian tumors express high levels of Wip1 nuclear protein (A&B). ERα$^{d/d}$ letrozole treated ovarian tumors have decreased Wip1 protein expression comparatively (C&D).

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